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(54) Title: ANTIBODIES AGAINST HYDROPHOBIC PROTEINS THAT ARE PROTECTIVE AGAINST CANDIDIASIS

(57) Abstract

Antibodies are disclosed that protect against candidiasis, particularly antibodies of the IgG class and antibodies that specifically bind to a hydrophobic protein antigen of the cell wall of a yeast from the *Candida* genus. Also disclosed are pharmaceutical compositions and therapeutic methods useful in the treatment of candidiasis as well as diagnostic methods.

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ANTIBODIES AGAINST HYDROPHOBIC PROTEINS THAT ARE PROTECTIVE AGAINST CANDIDIASIS

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RELATED APPLICATIONS

This application is related to U.S. provisional application Serial No. 60/122,216, filed March 1, 1999; U.S. provisional application Serial No. 60/120,764, filed February 19, 1999; U.S. provisional application Serial No. 60/120,765, filed February 19, 1999; and PCT Application US/00/_________ filed February 18, 2000, and based on the latter two U.S. provisional applications, all of which are herein incorporated by reference in their entirety.

FIELD OF THE INVENTION

The present invention relates to antibodies that protect a host against candidiasis, particularly to antibodies that specifically bind to hydrophobic proteins of the cell wall of a yeast from the *Candida* genus. The invention further relates to pharmaceutical compositions and therapeutic methods useful in the treatment of candidiasis and diagnostic methods useful in diagnosing candidiasis and monitoring the course of treatment of candidiasis.

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BACKGROUND OF THE INVENTION

The yeast genera, *Candida,* can cause a variety of clinical syndromes that are generically termed candidiasis and are usually categorized by the physiological site of involvement. The two most common syndromes are mucocutaneous candidiasis (*e.g.*, stomatitis or thrush, esophagitis and vaginitis) and invasive or deep organ candidiasis (*e.g.*, fungemia, endocarditis, and endophthalmitis). These syndromes are discussed in Dismukes, *Candidiasis,* IN CECIL'S TEXTBOOK OF MEDICINE 1827-1830 (Bennett *et al.* eds., 1996).

1). <u>Treatment of Candidiasis</u>:

Patients suffering from mucocutaneous infections may be treated with any one of several topical preparations including nystatin, clotrimazole, econazole, ketoconazole, butoconazole, terconazole, and miconazole. *Id.* The treatment for more clinically serious *Candida* related disease (*e.g.*, candidemia or disseminated candidiasis) includes administration of amphotericin B and fluconazole or both. Therapy for *Candida* peritonitis involves either intravenous amphotericin B or oral gluconazole. *Id.*

The medical literature also reported that various classes of antibodies (IgA, IgG and IgM) directed against several *C. albicans* antigens were of experimental interest and of diagnostic or therapeutic value. See, *e.g.*, Torres-Rodriguez *et al.*, 1997 *Mycoses* 40:439-44 with respect to *C. albicans* germ tube; and Reboli, 1993 *J. Clin. Microbiol.* 31: 518-23 with respect to a dot immunobinding assay involving total *Candida* protein. Monoclonal antibodies specific to an iC3b receptor, which is an integrin that has antigenic and structural homology with a *Candida* surface antigen, were demonstrated to increase survival of mice with disseminated candidiasis. Lee *et al.*, 1997 *Immunology* 92: 104-110. Similarly, antibodies to mannoprotein (MP) and aspartyl proteinase (Sap) have been shown to protect against vaginitis in rats. De Bernardis *et al.*, 1997 *Infect. Immun.* 65: 3399-3405.

U.S. Patents No. 4,670,382 and 4,806,465 to Buckley *et al.* (1989) describe IgG monoclonal antibodies against a set of closely related cytoplasmic antigens of *C.*

albicans, but present no therapeutic data showing efficacy against *Candida* infection. U.S. Patent No. 5,288,639 to Burnie *et al.* (1994) describes monoclonal antibodies against stress or heat shock proteins of *Candida*, which were shown to produce 33 % survival at 24 hours in animals challenged with a lethal dose of *C. albicans*. Also, U.S. Patent No. 5,641,760 to Yu *et al.* (1997) discloses monoclonal antibodies against *C. albicans* fimbrial subunits that are said to be useful for treating *C. albicans* infections. However, although this patent identifies antibodies as members of the IgG2 isotype, no *in vivo* data showing protection against *Candida* infection were provided.

Certain immunogenic phosphomannan preparations of *C. albicans*, which is known to contain adhesins, have been used to prepare vaccines for the treatment of, and elicit antibodies against, disseminated candidiasis due to infection by *C. albicans*. For example, European Patent No. 344,320 to Kawamura *et al.* (1989) describes human monoclonal antibodies of IgG and IgM classes that were raised against mannan extracted from *Candida*. Although antibodies of the IgG class are said to be preferred and agglutinating activity is discussed, the skilled artisan will understand that agglutination is distinct from protective effect and no therapeutic data against *Candida* infection were provided by Kawamura *et al.*

Therapeutic efficacy was shown in U.S. Patent No. 5,578,309 to Cutler *et al.* (1996) described the immunization of mice with liposome-encapsulated *Candida* phosphomannoprotein and obtained several monoclonal antibodies specific for that fraction. In addition, mice were immunized with a liposome encapsulated mannan adhesin extracted from the cell wall ("L-adhesin" or "L-mannan" or "L-mann"), and two IgM class monoclonal antibodies specific for yeast surface epitopes were described in Cutler *et al.* Although both antibodies (B6.1 and B6) were strong agglutinins, only one (B6.1) was shown to protect naive mice against disseminated candidiasis. Each antibody recognizes a distinct *C. albicans* mannan cell wall determinant, and the MAb B6.1 recognized a carbohydrate antigen. See, also, Han *et al.*, 1997 *Infect. Immun.* 65: 4100-07. The B6.1 antibody also enhanced ingestion and killing of yeast cells by polymorphonuclear leukocytes (PMNs) in the presence of serum complement. Caesar-TonThat *et al.*, 1997 *Infect. Immun.* 65: 5354-57.

2). Microbial Adhesion Molecules:

Fungal surface adhesins are critical virulence factors in mucocutaneous and disseminated mycotic disease because the transition from either normal flora status or an environmental saprobic state to pathogen status depends upon adhesion-mediated movement across host tissue barriers. Adhesion of microorganisms to host tissue is an initial step in the colonization of new environments. For certain organisms, such as the opportunistic fungal pathogen *Candida albicans*, adhesion to multiple host substrata contributes to dissemination of disease (Calderone & Scheld, 1987; Calderone *et al.*, 1994; Kennedy, 1990).

Cell surface hydrophobicity (CSH) plays an important role in the adhesive properties of C. *albicans* cells. Major shifts in antigenic display as revealed by changes in CSH occur during normal growth and morphogenesis of *C. albicans* in vitro (Hazen et al., 1988; Hazen, 1992) and in vivo (Glee et al., 1995). Hydrophobic cells bind more readily than hydrophilic cells to epithelial cells, endothelial cells, extracellular matrix proteins and plastics, and display more generalized binding to host tissues (Hazen, 1989; Hazen et al., 1991; Filler et al., 1996; Glee et al., 1996; Chaffin et al., 1998). Furthermore, hydrophobic cells demonstrate greater resistance than hydrophilic cells to phagocytic killing and have been shown to be more virulent in mice (Antley et al., 1988; Hazen *et al.*, unpublished results; Cutler et al., 1990). The expression of CSH by *C. albicans* promotes in vitro adhesion to epithelial cells and extracellular matrix proteins, lowers susceptibility to phagocytic killing and diversifies attachment sites in various diseases. These general features of CSH have been reviewed (Hazan et al., 1996; Glee et al., 1995). CSH therefore is an important factor in the development of disease caused by *C. albicans*.

Cell wall proteins are thought to contribute to, if not determine, the CSH status of *C. albicans* cells. Cell wall components contributing to fungal CSH are best known in filamentous organisms (Wessels et al., 1997) and partially known for yeasts (Hazen et al., 1996). Surface radiolabelling and hydrophobic interaction chromatography identified candidate hydrophobic proteins from the *C. albicans* cell wall as a limited number of small (<90kDa), minimally glycosylated proteins (Hazen et al., 1992; Hazen et al.,

1994). Hydrophobic proteins were identified and extracted from the *C. albicans* cell wall (Hazen *et al.*, 1990). Recent work demonstrated that these proteins are expressed on the surface of *C. albicans* cells *in vivo* (Glee *et al.*, 1995). The expression of cell surface hydrophobicity (CSH) by many medically important fungi is common (Hazen et al., 1996), indicating that cell wall hydrophobic proteins constitute important target molecules for prevention of fungal adherence and development of mycotic pathologies. Cell surface hydrophobicity status specifically influences the attachment of *Candida albicans* to various host tissue sites. For example, surface hydrophobicity is expressed in vivo during candidiasis (Glee et al.,1995). A relationship between CSH and attachment of *Candida* cells to extracellular matrix (ECM) proteins has also been shown (Silva et al., 1995). Hydrophobic proteins having molecular weights of 37, 38 and 40 kilodaltons have been shown to bind the ECM proteins fibronectin and laminin (Glee et al., 1996). Structural and functional evaluation of individual hydrophobic cell wall proteins would thus provide insight into the specific role of CSH in host-fungal interactions.

Iodine surface labeled wall proteins from hydrophilic and hydrophobic cells have been compared (Hazen *et al.*, 1990). This comparison identified a 38 kDa protein which appeared to be unique to hydrophobic cells (Hazen *et al.*, 1990) and which fell into the molecular mass range (30-40 kDa) of the hydrophobic cell wall proteins retained by Hydrophobic Interaction Chromatography (HIC)-HPLC (Hazen et al., 1994). Preliminary characterization of several of the hydrophobic proteins, including *C. albicans* 36, 38 and 40 kilodalton proteins, indicated that they have low levels of glycosylation which may influence CHS (Hazen & Glee, 1994). A further study revealed that changes in outer chain mannosylation, as opposed to complete loss of N-linked glycosyl groups, is sufficient to affect CSH (Masuoka and Hazen, 1997). In this study a monoclonal antibody designated as 6C5 that binds specifically to the 38 kilodalton cell wall protein of *C. albicans* was used to characterize the difference in cell surface mannosylation between *C. albicans* wild type strain and a variant thereof.

Additionally, the prior art describes several static adhesion assays for quantifying adherence of Candida to human endothelium (Filler et al., 1987; Mayer et al., 1992). Static adhesion assays in which anti-integrin antibodies or antibodies against non-

hydrophobic proteins that bind to host epithelial and endothelial cells were used to block binding of *Candida* spp. have also been described (Hostetter, 1994). However, none of the prior art establishes the adhesion activity of the hydrophobic cell wall proteins from *Candida* under physiologically relevant conditions present in fluid-tissue interfaces in vivo.

It remains a need in the art to identify hydrophobic proteins that participate in a dual virulence strategy of evading host immune mechanisms and diversifying adhesion interactions and to produce antibodies directed to these hydrophobic proteins capable of blocking binding of *Candida* cells to host tissue cells under physiologic shear. It also remains a need in the art to identify antibodies that recognize a hydrophobic protein that acts as a *Candida* adhesin and is therapeutically effective in inhibiting or preventing candidiasis.

SUMMARY OF THE INVENTION

The present invention relates to antibodies that protect a host against candidiasis, particularly disseminated candidiasis, mucocutaneous candidiasis (*e.g.*, stomatitis or thrush, esophagitis and vaginitis or vaginal candidiasis) and invasive or deep organ candidiasis (*e.g.*, fungemia, endocarditis, and endophthalmitis). More particularly, the invention relates to adhesion-blocking antibodies that specifically bind to epitopes on the hydrophobic cell wall proteins of the yeast *Candida*. The invention is also directed to hydrophobic cell wall proteins of the yeast *Candida* which interact with ligands from host tissue cells under simulated physiologic shear. The invention also relates to therapeutic methods useful in the treatment of candidiasis and diagnostic methods useful in diagnosing candidiasis and monitoring the course of treatment of candidiasis.

It is an object of the present invention to provide a monoclonal antibody that specifically binds to an epitope of a hydrophobic cell wall protein of a yeast from the *Candida* genus and inhibits the binding of the protein to a tissue of a mammalian host. Preferably the hydrophobic cell wall protein mediates adhesion of the yeast to the tissue, more preferably under conditions of physiological shear present in the tissue.

Hydrophobic proteins of particular interest are those having a molecular weight, as determined by HIC-HPLC, of less than about 90 kDa, more preferably in the range of about 20-70 kDa and more preferably about 37 kDa, about 38 kDa, about 40 kDa or about 41 kDa. 36. Preferred proteins are those that are capable of binding to the host tissue under physiological shear conditions present in the tissue.

Yet another object of the invention is to provide antibodies that bind to various species of yeast, including *C. albicans, C. kefyr*, *C. lipolytica*, *C. rugosa, C. stellatoidea* and *C. tropicalis* and strains thereof. Contemplated antibodies are of the classes IgG, IgA and IgM. It is a further object of the invention to provide particular monoclonal antibodies identified herein as 6C5, 5F8, 5D8, 1C1 antibodies and fragments or mixtures thereof.

Also, the antibody preferably binds to the yeast under conditions of physiological shear present in the tissue of a host. Preferably, the antibody is a human antibody, a chimeric antibody, or a humanized antibody. Also contemplated are the antigen binding fragment of the monoclonal antibodies, including a Fv fragment, a Fab fragment, a Fab' fragment, and a $F(ab')_2$. Contemplated are antibodies that are protective against disseminated *Candida* infection in the host or are protective against mucocutaneous *Candida* infection in the host. Preferred are antibodies that block attachment of a yeast to the host's tissue or cells. Contemplated tissues include endothelial cells, epithelial cells or extracellular matrix proteins.

It is a further object of the present invention to provide pharmaceutical compositions that comprise such antibodies, together with pharmaceutically acceptable carrier and excipients. Such formulations may be formulated for systemic administration, topical administration or as an aerosol. It also is contemplated that these pharmaceutical compositions may also include one or more other therapeutic agents, such as antifungal agent, including amphotericin B, fluconazole, new generation azoles and mixtures thereof.

Yet another aspect of the present invention relates to methods for treating candidiasis in a subject comprising the step of administering to a subject a therapeutically effective amount of the such pharmaceutical compositions. Particularly

contemplated are such methods which involve administering a therapeutically effective amount that is effective to inhibit the binding of a hydrophobic cell wall protein of a yeast from the *Candida* genus to a tissue of a mammalian host, to treat or prevent disseminated candidiasis or to treat or prevent mucocutaneous candidiasis.

In another aspect, the present invention relates to a diagnostic kit that includes the foregoing antibodies, together with a reagent for detecting binding of the antibody to a hydrophobic cell wall protein of a yeast from the *Candida* genus. Also contemplated are hybridoma cells that expresses such antibodies.

BRIEF DESCRIPTION OF THE DRAWING FIGURES

Fig. 1 shows a Western blot of *Candida albicans* cell wall digests. RCD samples from hydrophobic yeasts (1), germ tube initials (2) and hydrophilic yeasts (3) are shown. Proteins were visualized by their recognition by the polyclonal α-HICF6 antiserum or the monoclonal antibodies (5F8, 6C5 and 5D8). Arrowheads in the α-HICF6 section indicate the positions of the 37, 39. and 40 kDa. proteins.

Fig. 2 shows an indirect immunofluorescent staining of C. *albicans* germ tube initials by 6C5, 5D8 and 5F8. BF: Photomicrographs of cells using Nomarski optics. IEF: Immunofluorescence showing antibody binding as detected by FITC-labeled secondary antibody.

Fig. 3 shows a ligand blot of C. *albicans* cell wall digests. RCD samples from hydrophobic yeasts (1), germ tube initials (2) and hydrophilic yeasts (3) are shown. Fibronectin (Fn) or laminin (Ln) were used to probe for ECM binding proteins. Bound Fn or Ln was detected by α-Fn or α-Ln antibodies. Total protein was visualized by Amido Black staining (Amido). Arrowheads indicate the positions of the 37, 38 and 40 kDa proteins in each panel.

Fig. 4 shows inhibition of C. *albicans* adhesion to immobilized Fn (a, c) or Ln (b, d). Results of these experiments are presented in box plot form. The top and bottom of the outlined box represent the 75' and 25' precentile of the dataset, respectively. The interior horizontal line indicates the median. The area of grey shading indicates the 95 %

confidence interval about the median. Data sets with nonoverlapping confidence intervals were considered to be significantly different.

Fig. 5 shows MAB 6C5 inhibits hydrophobic yeast cell binding to activated HUVECs. Results of in vitro shear analysis show that MAB 6C5 pretreatment of C. *albicans* caused significant inhibition of binding to IL-1β activated endothelial cells compared to control yeast only ($P < .001$, t-test) and control MAB EL246 treatment ($P < .00\ 1$, t-test). The graph shows the heterotypic binding events (average number of foci/field) for the assay.

Fig. 6 shows the influence of other anti-hydrophobic antibodies on hydrophobic C. *albicans* binding to IL-1β activated HUVECs. A mixture of MAb 5F8, 5D8, and 1C1 ascites was compared with other antibody treatments for inhibition of hydrophobic yeast cell binding to activated HUVECs. MAb S10 was included as an irrelevant ascites control.

Fig. 7 shows the in vitro recirculating loop system used in the shear assay.

DETAILED DESCRIPTION OF THE INVENTION

Several groups have recently identified *C. albicans* cell wall proteins that bind to extracellular matrix (ECM) proteins, primarily through the use of affinity chromatography and western blotting (Bouchara *et al.*, 1990; Calderone *et al.*, 1988; Casanova *et al.*, 1992; Klotz *et al.*, 1993, 1994; Ippez-Ribot *et al.*, 1994; Saxena & Calderone, 1990). The ECM proteins form a substrata in host tissue which may provide binding sites for yeast adhesion. The identified ECM-binding proteins of *C. albicans* are of intermediate molecular mass (30-70 kDa), similar to that of the hydrophobic proteins. In addition, sequence analysis of the ECM proteins fibronectin (Fn) and laminin (Ln) has revealed hydrophobic regions surrounding the cell binding site. Also, recent work showed a relationship between CSH and attachment of *Candida* cells to ECM proteins (Silva *et al.*, 1995).

The present invention is based upon the discovery that distinct, exposed surfaces on the hydrophobic proteins of *Candida* are responsible for CSH status, and that these proteins are involved, at least in part, in adhesion of *Candida* cells to host tissue. The

role of these proteins in adhesion involves their interaction with the ECM proteins, and the present invention relates to an assessment of the interactions between the hydrophobic proteins and the ECM proteins which involve the hydrophobic regions on both sets of proteins. As discussed herein, these proteins serve functions relevant to pathogenesis in addition to contributing to the hydrophobic character of the cell wall. They contribute to pathogenesis by mediating attachment to host ECM proteins which are located throughout the vascular walls and interstitial sites.

The present invention further relates to immunological and biochemical analyses of three hydrophobic proteins (37, 38 and 40 kDa) initially selected because of their differential distribution among various pathogenic *Candida* species. In addition to results presented by others, previous work showed that proteins of this size bind fibronectin and laminin (Glee *et al.*, 1996). Initial characterization of the three proteins and assessment of their distribution in the *Candida* cell wall are described below.

HPLC-fractionated cell wall proteins were used to generate four monoclonal antibodies, 6C5, 5F8, 5D8, and 1C1, each of which recognizes a different hydrophobic protein. Using these monoclonal antibodies, it was determined that the four antigens are in the cell wall and on the surface, particularly on the surface of germ tubes. Soluble ECM protein bound to each hydrophobic protein and each monoclonal antibody was able to inhibit whole cell attachment to immobilized ECM. Thus, the results suggest that hydrophobic cells may adhere to host tissues via a contribution of hydrophobic cell wall protein attachment to ECM. If correct, then C. *albicans* possesses multiple surface molecules capable of ECM recognition.

Binding of microorganismal cell surface proteins to the extracellular matrix (ECM) is considered to play an important role in adhesion (for reviews see Calderone, 1993; Patti & Hook, 1994; Pendrak & Klotz, 1995; Roberts, 1990). C. *albicans* cells have been shown to bind to several ECM proteins in vitro and in vivo, including fibronectin (Jakab *et al.*, 1993; Scheld *et al.*, 1985; Skerl *et al.*, 1984), laminin (Bouchara *et al.*, 1990; Ippez-Ribot *et al.*, 1994), fibrinogen (Bendel *et al.*, 1993; Bouali *et al.*, 1986; Robert *et al.*, 1991), fibrin (Maisch & Calderone, 1980), and entactin (Lopez-Ribot & Chaffin, 1994). The cited studies have generally reported identification of individual

ECM binding proteins. Comparison of these reports reveals that several proteins of differing molecular masses can each bind the various ECM proteins. For example, *Candida* wall proteins of 37, 60, 62, 67, and 68 kDa have been observed to bind laminin (Bouchara *et al.*, 1990; Lopez-Ribot *et al.*, 1994). These molecular masses are very similar to those of proteins reported to bind fibrinogen (Casanova *et al.*, 1992), fibronectin (Klotz *et al.*, 1994), Od (Calderone *et al.*, 1988; Saxena & Calderone, 1990), and iC3b (Kanbe *et al.*, 1991). Although there is some evidence that this multiplicity is due to protein glycosylation (Kanbe *et al.*, 1991), it has not been demonstrated that these proteins are structurally related to each other.

This multiplicity of ECM binding proteins in the *Candida* cell wall is consistent with the results of Fn and Ln binding to RCD proteins (Fig. 3). Other investigators, describing purification of ECM-binding proteins, reported one or a few wall proteins which bound a given ECM protein. There are several possible explanations for this discrepancy. The main difference between the present study and those previously done is that the wall digest used here was not dialyzed. Previous work by our group showed that dialysis leads to the loss of the majority of protein, particularly the hydrophobic proteins (Glee *et al.*, 1996). Another factor which may be involved is the digest procedure itself. The β-1,3-glucanase used in the digest cleaves the wall glucan, but not into monosaccharides. Kapteyn, *et al.* have suggested that some of the cell wall proteins are covalently linked to the wall β-1,3-glucan via β-1,6-glucan and glycosyl phosphotidylinositol (Kapteyn *et al.*, 1994, 1995). Thus, the multiple electrophoretic mobilities may be due to the presence of oligoglucosides of varying length.

The ECM-binding proteins described here were first characterized based on their hydrophobicity rather than, as has been done most often, by direct functional assays. The shared hydrophobicity may provide evidence for the structural relatedness mentioned above. We hypothesize that the fungal cell wall proteins and the ECM proteins interact through cognate hydrophobic regions.

The peptide sequence RGD has been accepted as a cell binding site on ECM proteins (Ayad *et al.*, 1994). However, several groups have reported that RGD alone is not sufficient to significantly inhibit *C. albicans* binding to ECM and host cells (Bendel

et al., 1993; Klotz & Smith, 1991; Negre *et al.*, 1994; Ollert *et al.*, 1993). Klotz and Smith (1991) reported 11% inhibition of Fn binding to *C. albicans* yeasts by GRGDTP and no inhibition by GRGDSP, but 68 % inhibition by intact Fn. Similarly, results from Bendel *et al.* indicated that only peptides containing RGD along with oligomer flanking sequences were able to inhibit binding of C. *albicans* to epithelial cells (Bendel *et al.*, 1993). Furthermore, Negro *et al.* (1994) and Ollert *et al.* (1993) were able to show inhibition of binding by using peptides; or tryptic fragments that did not contain RGD, but did contain hydrophobic residues.

Other groups have commented on the overlap in biological activities or the apparent binding of many ligands by a single receptor (Bouchara *et al.*, 1990; Klotz & Smith, 1991; Lopez-Ribot *et al.*, 1994; Tronchin *et al.*, 1989). Recently, Bouchara *et al.* have extended these results and concluded that the Ln and fibrinogen receptors on *Aspergillus fumigatus* are identical (Bouchara *et al.*, 1997). The results presented here suggest that, beyond the RGD sequence, hydrophobicity is also a shared trait and may contribute to a common mechanism driving ECM-cell interactions. Since RGD is typically found on a loop, it is possible that the interaction between ECM proteins and the *Candida* cell wall proteins begins with contact at RGD and is driven to completion by hydrophobic chemistry. Alternatively, initial binding may take place between hydrophobic structures with RGD serving as a final locking mechanism. Preliminary experiments additionally suggest that some of the hydrophobic proteins are found in close proximity to each other, if not physically linked. It may be that ECM proteins need to recognize the hydrophobic site in the context of another structure, or that the hydrophobic site strengthens and enhances binding once initial interaction has taken place.

The involvement of cell surface hydrophobicity in adhesion extends to other microorganisms as well. Vázquez-Juárez, *et al.* reported the involvement of CSH in the attachment of yeast strains to trout intestine (Vázquez-Juárez *et al.*, 1997). Davies, *et al.* (Davies *et al.*, 1996) showed that there is a hydrophobic interaction between the bacterium *Pasteuria penetrans* and its host nematode. Courtney, *et al.* showed that

fibronectin interacts hydrophobically with the Streptococcus cell surface (Courtney *et al.*, 1990).

Studies using the isolated proteins and peptides will allow more detailed characterization of the ECM-*Candida* protein interaction and lead to a better understanding of the interaction of hydrophobic proteins with ECM proteins, and the role of hydrophobic proteins in fungal adhesion and pathogenesis. Understanding the role that cell surface proteins play in conferring CSH status may also lead to an understanding of this common trait in microbial adhesion.

1. *Definitions*

The term "antibody" as used herein, unless indicated otherwise, is used broadly to refer to both antibody molecules and a variety of antibody-derived molecules. Such antibody-derived molecules comprise at least one variable region (either a heavy chain or light chain variable region) and include molecules such as Fab fragments, Fab' fragments, $F(ab')_2$ fragments, Fv fragments, Fabc fragments, single chain Fv (scFv) antibodies, individual antibody light chains, individual antibody heavy chains, chimeric fusions between antibody chains and other molecules, and the like.

Antibodies of the invention may be isolated from a hybridoma cell, the serum of a vertebrate, recombinant eukaryotic or prokaryotic cells transfected with a nucleic acid encoding the antibody, which may include plant cells, ascites fluid, or the milk of transgenic animals.

The term "antigen" means a molecule that is specifically recognized and bound by an antibody. The specific portion of the antigen that is bound by the antibody is termed the "epitope".

The term "humanized antibody" refers to an antibody which is substantially human in structure; that is, it derives at least substantially all of its constant regions from a human antibody even though all or a part of its variable regions are derived from some other species. "Human antibody" refers to an antibody which is encoded by a nucleotide of human origin and such nucleotides may be modified by the skilled artisan by known nucleotide manipulation techniques.

Antibodies described herein also may contain alterations of the amino acid sequence compared to a naturally occurring antibody. In other words, the antibodies of the invention need not necessarily consist of the precise amino acid sequence of their native variable region or constant region framework, but contain various substitutions that improve the binding properties of the antibody to its cognate antigen or change the binding of the antibody to effector molecules such as complement or the Fc receptor. In another format, a minimal number of substitutions are made to the framework region in order to ensure reduced, and preferably, minimal immunogenicity of the antibody in humans. In preferred embodiments of recombinant antibodies of the invention, any non-human framework regions used may be altered with a minimal number of substitutions to the framework region in order to avoid large-scale introductions of non-human framework residues.

The term "conventional molecular biology methods" refers to techniques for manipulating polynucleotides that are well known to the person of ordinary skill in the art of molecular biology. Examples of such well known techniques can be found in MOLECULAR CLONING: A LABORATORY MANUAL 2ND EDITION, Sambrook *et al.*, Cold Spring Harbor, N.Y. (1989) and in CURRENT PROTOCOLS IN MOLECULAR BIOLOGY" ________________. Examples of conventional molecular biology techniques include, but are not limited to, *in vitro* ligation, restriction endonuclease digestion, polymerase chain reaction (PCR), cellular transformation, hybridization, electrophoresis, DNA sequencing, cell culture, and the like.

The term "isolated" or "substantially pure" as used herein refers to an antibody or, for example, a fragment thereof, which is substantially free of other antibodies, proteins, lipids, carbohydrates or other materials with which it is naturally associated. One skilled in the art would be able to isolate or to substantially purify the antibodies described herein using conventional methods for antibody or protein purification.

The terms "protective" or "therapeutically effective" generally mean that the antibody is effective to block attachment of a yeast cell to its target tissue or cells in a host, or to decrease or prevent the increase in fungal cell levels in the bloodstream or at an organ site or other site of infection. More specifically, the phrase "protective" or

"therapeutically effective" means that the antibodies or pharmaceutical compositions according to the present invention are able opsonize *Candida* pathogens to facilitate macrophage, monocyte or neutrophil phagocytosis and killing, or can activate the macrophages that can amplify the cellular and immune responses. Preferably, the treatment methods of the present invention are effective to kill at least about 20 %, more preferably 40%, even more preferably 60% and most preferably 90% or more of the *Candida* organisms in an infected mammalian host in a therapeutic course of treatment.

The terms "shear force" means the capacity to deform an elastic body by producing an opposite but parallel sliding motion of the body in the body's plane. For example, a pathogen that has adhered to a substrate in an elongated tube experiences a shear force from the fluid flowing past the pathogen that tends to move the pathogen in the direction of the fluid flow. In a related aspect, the phrase "substantially the physiological equivalent" when referring to shear force, means a shear force that is similar to the shear force naturally produced in various tissues or organs which experience time variant flow, such as the vasculature and cardiac muscle.

The terms "variable region" and "constant region" as used herein in reference to antibody and immunoglobulin molecules have the ordinary meaning given to the term by a person of ordinary skill in the art of immunology. Both antibody heavy chains and antibody light chains may be divided into a "variable region" and a "constant region." The point of division between a variable region and a contrast region may be determined by the person of ordinary skill in the art by reference to standard texts describing antibody structure. See, *e.g.*, Kabat *et al.*, "Sequences of Proteins of Immunological Interest: 5th Edition" U.S. Department of Health and Human Services, U.S. Government Printing Office (1991).

2. *Candida Related Conditions*

Among the more than 150 recognized species of *Candida, C. albicans* is the most commonly identified pathogen in humans. Other clinically important species include *C. guilliermondi, C. krusei, C. parapsilosis, C. pseudotropicalis* and *C. tropicalis*. Mucocutaneous infections include thrush or oropharyngeal candidiasis, cheilosis,

esophagitis, gastrointestinal candidiasis, intertrigo, paronychia, vulvovaginitis, balanitis, *Candida* cystitis, and chronic mucocutaneous candidiasis. Numerous diagnostic categories exist for serious or deep *Candida* infection including candidemia, disseminated candidiasis, systemic candidiasis, invasive candidiasis, visceral candidiasis and terms indicating involvement of specific organs such as hepatosplenic candidiasis and ocular candidiasis. See, *e.g.*, Dismukes, 1996. Serious or deep *Candida* infections are frequently observed in immunodeficient or immune compromised patients, such as in patients with Acquired Immundeficiency Syndrome (AIDS).

3. *Preparation of Hybridomas that Produce Monoclonal Antibodies Against Candida antigens.*

To produce antibodies, various species of host animals may be immunized by injection with the hydrophobic protein antigens described herein or with appropriately prepared *Candida* extracts or whole cells. Appropriate animals for this purpose include, but are not limited to rabbits, mice, and rats, etc. Various adjuvants may be used to increase the immunological response, depending on the host species, including but not limited to Freund's (complete and incomplete), mineral gels such as aluminum hydroxide, surface active substances such as lysolecithin, pluronic polyols, polyanions, peptides, oil emulsions, keyhole limpet hemocyanin, dinitrophenol, and potentially useful human adjuvants such as BCG (bacilli Calmette-Guerin) and *Corynebacterium parvum*.

Monoclonal antibodies to *Candida* antigens may be prepared by using any technique which provides for the production of antibody molecules by continuous cell lines in culture. These include but are not limited to the hybridoma technique originally described by Kohler and Milstein (*Nature*, 1975, 256:495-497), the human B-cell hybridoma technique (Kosbor *et al.*, 1983, *Immunology Today*, 4:72; Cote *et al.*, 1983, *Proc. Natl. Acad. Sci.*, 80:2026-2030) and the EBV- hybridoma technique (Cole *et al.*, 1985, Monoclonal Antibodies and Cancer Therapy, Alan R. Liss, Inc., pp. 77-96). In addition, techniques developed for the production of "chimeric antibodies" (Morrison *et al.*, 1984, *Proc. Natl. Acad. Sci.*, 81:6851-6855; Neuberger *et al.*, 1984, *Nature*,

312:604-608; Takeda *et al.*, 1985, *Nature*, 314:452-454) by splicing the genes from a mouse antibody molecule of appropriate antigen specificity together with genes from a human antibody molecule of appropriate biological activity can be used. Alternatively, techniques described for the production of single chain antibodies (U.S. Pat. No. 4,946,778) can be adapted to produce *Candida* specific single chain antibodies.

4. *Isolation of Candida antigen specific B cells.*

Antigen specific B cells may be isolated from convenient samples, such as peripheral blood lymphocytes from a human donor, either normal or a patient infected with *Candida*, by techniques known and available in the art. For instance, fusion proteins of the invention may be used to detect and isolate B cells which express immunoglobulin which specifically binds to the hydrophobic protein antigens described herein by affinity chromatography, fluorescent activated cell sorting (FACS) and other commonly used techniques such as Zn-chelating sepharose or protein-A sepharose (see Harlow *et al.*, ANTIBODIES: A LABORATORY MANUAL, Cold Spring Harbor Laboratory, 1988).

As another example, lymph nodes obtained from a candidiasis patient or normal individual may be cut into fine pieces and meshed through a wire gauze using a rubber policeman. Pure B cells may be isolated using CD19 coated immunomagnetic beads. Antigen specific B cells may be isolated using the appropriate fusion protein by affinity chromatography or fluorescent activated cell sorting. The *Candida* antigen specific B cells may then be immortalized using known techniques such as immortalization by EBV. Any effective lymphotropic virus or other transforming agent able to transform the B-cells to grow in continuous culture and still produce monoclonal antibodies specific for the *Candida* associated antigens can be used.

5. *Isolation of antigen specific immunoglobulin heavy and light chain sequences.*

In addition to providing *Candida* hydrophobic protein specific antibodies, the subject invention provides for polynucleotides encoding *Candida* specific antibodies. The polynucleotides may have a wide variety of sequences because of the degeneracy of

the genetic code. A person of ordinary skill in the art may readily change a given polynucleotide sequence encoding a *Candida* specific antibody according to the present invention into a different polynucleotide encoding the same antibody. For example, the polynucleotide sequence encoding the antibody may be varied to take into account factors affecting expression such as codon frequency, RNA secondary structure, and the like.

6. *Production of recombinant human antibodies*

The antibodies of the subject invention may be produced by a variety of methods useful for the production of polypeptides, *e.g.*, in vitro synthesis, recombinant DNA production, and the like. Preferably, humanized antibodies are produced by recombinant DNA technology. The antigen specific antibodies of the invention may be produced using recombinant immunoglobulin expression technology. The recombinant production of immunoglobulin molecules, including humanized antibodies is described in U.S. Patent No. 4,816,397 (Boss *et al.*), U.S. PATENT No. 4,816,567 (Cabilly *et al.*), U.K. patent GB 2,188,638 (Winter *et al.*), and U.K. patent GB 2,209,757. Techniques for the recombinant expression of immunoglobulins, including humanized immunoglobulins, can also be found, among others, in Goeddel *et al.*, "Gene Expression Technology Methods" IN ENZYMOLOGY Vol. 185 Academic Press (1991), and Borreback, ANTIBODY ENGINEERING, W. H. Freeman (1992). Additional information concerning the generation, design and expression of recombinant antibodies can be found in Mayforth, DESIGNING ANTIBODIES, Academic Press, San Diego (1993).

As an example, the recombinant antibodies of the present invention may be produced by the following process:

a) constructing, by conventional molecular biology methods, an expression vector comprising a nucleotide sequence that encodes an antibody heavy chain in which the CDRs and a minimal portion of the variable region framework that are required to retain donor antibody binding specificity are derived from the human immunoglobulin, and the remainder of the antibody is derived from another human immunoglobulin, thereby producing a vector for the expression of a humanized antibody heavy chain;

b) constructing, by conventional molecular biology methods, an expression vector comprising a nucleotide sequence that encodes an antibody light chain in which the CDRs and a minimal portion of the variable region framework that are required to retain donor antibody binding specificity are derived from the human immunoglobulin, and the remainder of the antibody is derived from another human immunoglobulin, thereby producing a vector for the expression of humanized antibody light chain;

c) transferring the expression vectors to a host cell by conventional molecular biology methods to produce a transfected host cell; and

d) culturing the transfected cell by conventional cell culture techniques so as to produce recombinant antibodies.

Host cells may be cotransfected with two expression vectors of the invention, the first vector encoding a heavy chain derived polypeptide and the second encoding a light chain derived polypeptide. The two vectors may contain different selectable markers but, with the exception of the heavy and light chain coding sequences, are preferably identical. This procedure provides for equal expression of heavy and light chain polypeptides. Alternatively, a single vector may be used which encodes both heavy and light chain polypeptides. The coding sequences for the heavy and light chains may comprise cDNA or genomic DNA or both.

The host cell used to express the recombinant antibody of the invention may be a bacterial cell such as *Escherichia coli,* or antigen binding fragments may be expressed in available phage display systems (see Winter *et al.* (1994) *Ann. Rev. Immunol.* 12: 433-455 and Little *et al.* (1995) *J. Biotechnol.* 41(2-3): 187-195). Preferably a eukaryotic cell or most preferably a mammalian cell, such as a Chinese hamster ovary cell, may be used. The choice of expression vector is dependent upon the choice of host cell, and may be selected by a person skilled in the art so as to have the desired expression and regulatory characteristics in the selected host cell.

The general methods for construction of the vector of the invention, transfection of cells to produce the host cell of the invention, and culture of cells to produce the antibody of the invention, are all conventional molecular biology methods. Likewise, once produced, the recombinant antibodies of the invention may be purified by standard

procedures of the art, including cross-flow filtration, ammonium sulphate precipitation, affinity column chromatography, gel electrophoresis and the like.

7. *Preparation of diagnostic, therapeutic and prophylactic compositions*

The antibodies of the present invention may be used in conjunction with, or attached to other antibodies (or parts thereof) such as human or humanized monoclonal antibodies. These other antibodies may be reactive with other markers (epitopes) characteristic for the disease against which the antibodies of the invention are directed or may have different specificities chosen, for example, to recruit molecules or cells of the human immune system to the diseased cells. The antibodies of the invention (or parts thereof) may be administered with such antibodies (or parts thereof) as separately administered compositions or as a single composition with the two agents linked by conventional chemical or by molecular biological methods. Additionally the diagnostic and therapeutic value of the antibodies of the invention may be augmented by labeling the humanized antibodies with labels that produce a detectable signal (either *in vitro* or *in vivo*) or with a label having a therapeutic property. Some labels, *e.g.*, radionuclides may produce a detectable signal and have a therapeutic property. Examples of radionuclide labels include ^{125}I and ^{131}I. Examples of other detectable labels include a fluorescent chromophore such as fluorescein, phycobiliprotein ortetraethyl rhodamine for fluorescence microscopy, an enzyme which produces a fluorescent or colored product for detection by fluorescence, absorbance, visible color or agglutination, which produces an electron dense product for demonstration by electron microscopy; or an electron dense molecule such as ferritin, peroxidase or gold beads for direct or indirect electron microscopic visualization. Labels having therapeutic properties include drugs for the treatment of candidiasis such as are described below.

The subject invention also provides for a variety of methods for treating and/or detecting *Candida* cells. These methods involve the administration to a patient of *Candida* specific antibodies, either labeled or unlabeled. One method of detecting *Candida* cells in a human involves the step of administering a labeled *Candida* specific antibody (labeled with a detectable label) to a human and subsequently detecting bound

labeled antibody by the presence of the label. Alternatively, the *Candida* specific antibodies may be linked or conjugated to a therapeutic molecule such as ricin or other toxins.

The recombinant antibodies of this invention may also be used for the selection and/or isolation of human monoclonal antibodies, and the design and synthesis of peptide or non-peptide compounds (mimetics) which would be useful for the same diagnostic and therapeutic applications as the antibodies (*e.g.*, Saragovi *et al.*, 1991 *Science* 253: 792-795).

When the *Candida* specific antibodies of the invention are used *in vivo*, the antibodies are typically administered in a composition comprising a pharmaceutical carrier. A pharmaceutical carrier can be any compatible, non-toxic substance suitable for delivery of the monoclonal antibodies to the patient. Sterile water, alcohol, fats, waxes, and inert solids may be included in the carrier. Pharmaceutically accepted buffering agents or dispersing agents may also be incorporated into the pharmaceutical composition.

The antibody compositions of the invention may be administered to a patient in a variety of ways. Preferably, the compositions may be administered parenterally, *i.e.*, subcutaneously, intramuscularly or intravenously. Aerosol formulations are also expressly contemplated. Injectable forms of administration are sometimes preferred for maximal systemic effect against systemic infections and infections of the respiratory tract and the deep tissues. When long term administration by injection is necessary, medi-ports, in-dwelling catheters or automatic pumping mechanisms may be used. Thus, this invention provides compositions for parenteral administration which comprise a solution of the human antibody or a cocktail thereof dissolved in an acceptable carrier, preferably an aqueous carrier. A variety of aqueous carriers can be used, *e.g.*, water, buffered water, 0.4% saline, 0.3% glycine and the like. These solutions are sterile and generally free of particulate matter. These compositions may be sterilized by conventional, well-known sterilization techniques.

The compositions may contain pharmaceutically acceptable auxiliary substances as required to approximate physiological conditions such as pH adjusting and buffering

agents, toxicity adjusting agents and the like, for example sodium acetate, sodium chloride, potassium chloride, calcium chloride, sodium lactate, *etc.* The concentration of antibody in these formulations can vary widely, *e.g.*, from less than about 0.5%, or at least about 1% to as much as 15 or 20% by weight and will be selected primarily based on fluid volumes, viscosities, *etc.*, in accordance with the particular mode of administration selected.

A preferred dose of antibody for systemic administration of the antibodies of the present invention is in the range of about 0.1 to about 5 mg/kg of body weight. A more preferred dose is in the range of about 0.5 to about 2.0 mg/kg, most preferably about 1.0 to about 1.5 mg/kg. Human or other mammalian subjects are treated with multiple doses of antibody pharmaceuticals on an appropriate schedule, for example, a schedule that results in and maintains substantially saturating antibody levels or significant opsonization levels in the blood or infected tissue of a patient undergoing treatment according to the methods of the present invention. For example, a one-time dose of a chimeric antibody may be administered as described in Clark *et al.*, " Effect of a chimeric antibody to tumor necrosis factor-(alpha) on cytokine and physiologic responses in patients with severe sepsis - A randomized, clinical trial" in *Crit. Care Med.* 26:1650-59 (1998).

Local or mucocutaneous infections would be treated by topical application of the therapeutic antibody compositions of the present invention. For oral delivery, for example, the pharmaceutical compositions may be administered in the form of a cream or a wash that can be applied by, *e.g.*, swab or by rinsing at period intervals. These compositions also may be formulated into buccal suppositories for release, *e.g.*, from the oral region over an extended period of time. In an alternative embodiment, tablets or oral insert or gum may be utilized as delivery vehicles. For vaginal delivery, the composition may be administered in a cream formulation, vaginal suppository or insert, as is well known in the art.

Pharmaceutically effective amounts would be those amounts of the proposed pharmaceutical compositions required to yield a positive effect. Positive effects include a reduction of organism load in the subject, death or inactivation of the organism, or

complete or nearly complete elimination of the infecting organism from the body. Preferably, the patient has an infection as measured by any appropriate testing parameter, which is reduced at least 100-fold, more preferably 1,000-fold, and even more preferably is undetectable after treatment.

Yet other embodiments of the invention are directed to compositions of the invention which can be used in combination with other agents (*e.g.*, anti-fungal agents) to maximize the effect of the compositions in an additive or synergistic manner. Agents that may be effective in combination with the compositions of the invention include other drugs and treatments which are known or suspected to have a positive effect against a *Candida* organism. Such agents include, but are not limited to, flucytosine, mycoconazole, fluconazole, itraconazole, ketoconazole, griseofulvin, amphotericin B, sulfdiazine, penicillin, chlortetracycline, chloramphenicol, streptomycin and other sulfonamides, and derivatives, modifications and combinations of these agents. Other agents are described, for example, in U.S. Patent No. 5,679,648 to McCaffrey *et al.* (1977).

Actual methods for preparing parenterally administrable compositions and adjustments necessary for administration to subjects will be known or apparent to those skilled in the art and are described in more detail in, for example, REMINGTON'S PHARMACEUTICAL SCIENCE, 15th Ed., Mack Publishing Company, Easton, Pa. (1980), which is incorporated herein by reference.

8. <u>*Diagnostic kits for detecting diseased tissues and Candida cells*</u>

A kit can be prepared that comprises an antibody according to the present invention capable of binding to a diseased tissue or to *Candida*. These kits can be used in conjunction with existing histological staining techniques to determine more quickly, as well as more accurately, what disease is present and the extent of infection or stage of disease. This would be useful for purposes of diagnosing, detecting and/or determining what therapy or therapies may be appropriate in treating a particular subject's disease.

The preferred kit would have the antibody prepared for contact with a tissue or biological fluid sample, for example. The sample then would be incubated with the

antibody, as would be known for conventional methods used in the art. After incubation with kit antibody, the cells and/or tissue would be examined for the presence or absence of binding. Standard assays to be used in such kits include, but are not limited to latex agglutination, radio immunoassay (RIA), enzyme-linked immunosorbent assay (ELISA) or other suitable antigen detection system.

In light of the foregoing general discussion, the specific examples presented below are illustrative only and are not intended to limit the scope of the invention. Other generic configurations will be apparent to one skilled in the art.

Examples

Experiment 1: Initial Characterization of Proteins and Assessment of their Distribution in the Candida Cell Wall

A). Protein reagents: Human fibronectin (hFn) and rabbit α-hFn were obtained from Promega. Mouse laminin (mLn, ultrapure) and rabbit α-mLn were purchased from Collaborative Biomedical Products (Becton Dickinson). Rabbit IgG, mouse IgG, mouse IgM, and alkaline phosphatase (AP)-conjugated goat α-mouse IgG were purchased from Sigma Chemical Co. AP-conjugated goat α-mouse IgM and AP-conjugated goat α-rabbit IgG were obtained from Jackson ImmunoResearch Laboratories. Fluorescein isothiocyanate (FITC)conjugated goat α-mouse IgG and FITC-conjugated goat α-mouse IgM were purchased from Cappel.

B). Strains and culture conditions: C. *albicans* isolates were cultured as previously described (Hazen & Hazen, 1987a, 1993). Briefly, cells were grown to stationary phase in phosphate-buffered (pH 7.2) yeast nitrogen base (plus amino acids, Difco Laboratories, Detroit, MI) containing 2 % (w/v) glucose (YNB2G). Cells grown to stationary phase at 23°C were hydrophobic (CSH ≥ 95 %); those grown to stationary phase at 37°C were hydrophilic (CSH ≤ 5%).

Seven species of *Candida* were compared for similarities in hydrophobic protein complement. C. *albicans* LGH1095 (the strain used for protein characterization. studies)

and LGH870 have already been described (Antley & Hazen, 1988). *Candida kefyr*, *C. krusei*, *C. parapsilosis*, and *C. tropicalis* isolates were clinical specimens isolated in the University of Virginia Medical Center Clinical Microbiology Laboratory. *Candida glabrata* ZB5 was taken from our frozen stock collection. Other isolates were obtained from the American Type Culture Collection (ATCC). Isolates were subcultured three times at 37°C in YNB2G.

Stationary phase yeast cells from cultures incubated at 23°C were germinated (2 h, 37°C, 1.0 X 10^{-6} cells per ml) in Auto-Pow minimal essential medium with Earle's salts, pH 6.8 (Flow Laboratories) supplemented with biotin (250 mg per liter), glucose (9 g per liter), glycine (1 g per liter) and HEPES (6 g per liter) as previously described (Hazen & Hazen, 1987b). Only hydrophobic stationary phase yeast cells were induced to germinate because germination of hydrophilic yeasts produces predominantly pseudohyphae rather than true germ tubes.

C). Release and isolation of cell wall proteins: Proteins were released from the walls of stationary phase yeast and germinated cells by the previously described rapid crude digest (RCD) procedure. (Glee *et al.* 1995) Digestion was stopped by centrifugation (2 x 10 min. at 14,000 g) and removal of the supernatant fluid when the concentration of released protein reached 300-500 mg per ml (Coomassie Plus assay, Pierce Chemical Co.). Protease inhibitors were replenished in the final supernatant fluid and the final protein concentration was determined (bicinchoninic acid protein assay, Pierce) (Smith *et al.*, 1985). This method of digestion minimizes cytoplasmic contaminants based on the lack of ghost cells present at the end of the digestion period. The RCD material was not dialyzed, as previous work from our laboratory showed that the majority of wall protein is lost during dialysis (Glee *et al.*, 1996).

D). Polyclonal and monoclonal antibody production: Separation of the RCD proteins by hydrophobic interaction chromatography (HIC-HPLC), and the production of a polyclonal antiserum (designated α-HICF6 pAb) against the hydrophobic HIC fractions (6 and 7) has been previously described (Glee *et al.*, 1995; Hazen & Hazen, 1992).

Monoclonal antibodies (MAb) to C. *albicans* hydrophobic proteins were produced in collaboration with the University of Virginia Medical Center Hybridoma Facility (Chang *et al.*, 1994). A/J or BALB/c mice (maintained in an American Association for the Accreditation of laboratory Animal Care approved facility) were immunized with hydrophobic proteins from HIC-HPLC fractions 6 and 7 as previously described (Glee *et al.*, 1995). Antiserum reactivity was monitored by western blot analysis of C. *albicans* hydrophobic proteins (HIC-HPLC fractions 6 and 7). A final intrasplenic boost of approximately 3 μg of gel-purified (Glee *et al.*, 1996) 32-40 kDa proteins from HIC-HPLC fraction 6 was administered (Spitz *et al.*, 1984). Splenocytes were fused with Sp/O myeloma cells, plated in 96-well microtiter plates, and the supernatant fluids screened for antibody by enzyme-linked immunosorbent assay (ELISA). Positive wells were screened by western blot analysis of combined HIC-HPLC fraction 6 and 7 proteins. Reactive hybridomas were subcloned and retested by western blot. Monoclonal antibodies were evaluated for isotype (Pharmingen). The three hybridomas chosen for further study were designated 6C5-H4CA (6C5, derived from A/J mice), 5D8-AI2CA (5D8, derived from BALB/c mice) and 5F8-ElOCA (5F8, derived from BALB/c mice). Ascitic fluid was produced in BALB/c mice.

E). Electrophoresis and western blotting: Cell wall proteins from RCD were separated by SDS-PAGE using 12.5 % (w/v) acrylamide resolving gels. Cell wall proteins were also separated by preparative isoelectric focusing (Rotofor, Bio-Rad). Ampholytes, (PH 4-7, BioLytes, Bio-Rad) were mixed with the RCD solution to a final concentration of 2% (w/v), The solution was introduced into the focusing chamber and electrophoresed according to the manufacturer's specifications. The fractions were harvested following focusing and loaded onto a SDS-PAGE slab gel as above. Following electrophoresis, the separated proteins were transferred to nitrocellulose (BA-85, Schleicher and Schuell) membranes as described (Glee *et al.* , 1995).

Strips were cut from the membranes, rehydrated with water and soaked in Dulbecco's PBS, pH 7.2. Strips were blocked by incubation (37 °C, 1 h) in Dulbecco's PBS containing 5% (w/v) dry nonfat milk and 0.2% (v/v) Tween 20. This solution was also

used for all antibody dilutions and wash steps. Blocked strips were incubated (37 °C, 1-2 h) in primary antibody solution. 6C5 was used at a 1:2000 dilution of ascites. 5F8 and 5D8 were used as the hybridoma culture supernatant fluid without dilution. The polyclonal antiserum, α-HICF6 pAb, was used at a 1: 1000 dilution. Strips were washed three times (10 min each) and incubated (37 °C, 1 h) in secondary antibody solution. Secondary antibodies were alkaline phosphatase (AP)-conjugated goat α-mouse IgG (1:500) to recognize 6C5 or 5D8, and conjugated goat α-mouse IgM (1:500) for 5F8. Final washes and detection were as described (Glee *et al.*, 1995). A mixture of the two secondary antibodies was used for lanes treated with the polyclonal antiserum.

For ligand blot analysis, mLn or hFn were diluted to 50 μg per ml in blocking solution and incubated (37°C, 3 h) with blotted RCD proteins. The blot strips were washed three times (10 min each) and incubated (37 °C, 1 h) with rabbit α-mLn or rabbit α-hFn (1: 1000). The strips were washed as before and incubated with AP-conjugated goat α-rabbit IgG (37°C, 1 h). Final washes and detection were as described (Glee *et al.*, 1995).

F). Immunofluorescence: For immunofluorescence (IFA) experiments, stationary phase yeast cells (26 h) or germinated yeast cells were harvested, washed, and fixed in 1 % (v/v) formaldehyde for 1 h at room temperature. Fixed cells (250 μl) were placed in a microcentrifuge tube. The suspension was centrifuged and the supernatant fluid was removed. The pellet was suspended in 100 μl of primary antibody diluted in DPBS containing 10% (v/v) normal goat serum (Jackson ImmunoReasearch Laboratories) and the suspension was incubated at room temperature for 1 h. 6C5 was used at a 1: 100 dilution of a serum free preparation. 5F8 and 5D8 were used at a 1: 100 dilution of ascites. The cells were then washed three times with 250 μl of cold DPBS. The final pellet was suspended in 150 μl of secondary antibody diluted in DPBS containing 10% (v/v) normal goat serum and the suspension was incubated as before. The secondary antibody for 6C5 and 5D8 was FITC-conjugated goat a-mouse IgG (1:50). For 5F8, the secondary antibody was FITC-conjugated goat α-mouse IgM (1:50). The cells were

washed as before and the final pellet suspended in 100 μl of 0. 1 % (w/v) p--phenylenediamine in 90% (v/v) glycerol.

G). Cell adhesion assay: Cell adhesion assays were carried out in 48-well, flat-bottom, non-tissue culture treated, polystyrene multiwell plates (Falcon #1178). Fn and Ln were diluted to 66.7 μg/ml in DPBS. The distilled water and all buffer solutions used in this assay were sterilized by autoclaving. The wells were incubated with 150 μl of the diluted ECM protein solution (10 μg protein) overnight at 4 °C. The wells were then washed three times with cold DPBS.

The wells were blocked with 250 μl of DPBS containing immunoglobulin. The immunoglobulin used for blocking was purified from normal mice, used at the same concentration as the test antibody, and of the same isotype as the test antibody unless otherwise noted. The wells were incubated in blocking solution for 2 h at room temperature. During the block incubation, cells from a third transfer (23 °C) culture were harvested by centrifugation and washed twice with cold distilled water. If required, germination was initiated at this point, after which the germ tube initials were harvested and washed as above. Cell concentration and CSH were determined and, in the case of germ tubes, percent germination. From the washed cell suspension, 3×10^6 cells were transferred to each of several glass tubes containing 2 ml of treatment solution.

For treatments using 6C5 and 5D8, cells were added to a solution of ascites diluted (1:300 and 1:500, respectively) in DPBS. The final concentration of antibody was 12 μg/ml. For experiments using 5F8, cells were added to a solution of ascites diluted 1:300 in DPBS (23 μg/ml). Irrelevant mouse IgG2a and IgM were used as controls for 6C5/5D8 and 5F8, respectively, at the same concentration. Cells were incubated in the antibody solution at room temperature for 15 minutes, centrifuged and washed (once) with DPBS. Following treatment, a final cell count was performed and the cell concentration adjusted to 8×10^2 cells per ml, if necessary.

Immediately before the cells were added to the wells, the blocking solution was removed and the wells gently washed once with DPBS. Following the wash, 200 cells from each treatment were added to their respective wells. The plates were then incubated

at 37°C for 15 min. The wells were gently washed three times with DPBS to remove non-adherent cells and covered with 300 μl of 1.7 % (w/v) molten corn meal agar (CMA, Difco) previously tempered at 45°C. The agar was allowed to solidify and the plates were incubated overnight at 37°C. A replica aliquot of 200 cells from each treatment was used to inoculate a CMA plate. These plates were also incubated overnight at 37 °C. Percent adhesion was calculated by: (colonies per well / colonies per CMA plate) X 100. All samples were done in duplicate.

The design of the cell adhesion experiments is significant. First, the ECM proteins are used in a polymerized, immobilized state, as would be found in the ECM of the host/patient. Second, the proteins are exposed or expressed mostly on germ tubes (as discussed below).

Experiment 2: **Characterization of the monoclonal antibodies**

Results of isotyping indicated that 6C5 and 5D8 are IgG2a and 5F8 is IgM. All three antibodies are κ haplotype. Western blots of RCD proteins from hydrophobic cells were probed with the MAb. 6C5 recognizes a protein with an approximate molecular mass of 38 kDa (Fig. 1). The protein recognized by 5F8 is approximately 40 kDa molecular mass (Fig. 1). The protein recognized by 5D8 is approximately 37 kDa molecular mass (Fig. 1). Separation of RCD proteins based on isoelectric point (Rotofor, Bio-Rad) and subsequent western blot analysis indicated that the 37, 38 and 40 kDa proteins have an approximate pI of 6.2, 7.3, and 6.4, respectively. Proteins of these molecular masses were also among the RCD proteins recognized by a polyclonal serum generated against the hydrophobic wall proteins (Fig. 1). The MAb were used to probe blots of HIC-HPLC fractions. Results showed that the antigens for the MAb were present only in HIC-HPLC fractions 5-7, with the greatest concentration in fraction 6 (not shown). The latter results indicate that the proteins recognized by 6C5, 5D8 and 5F8 are in the hydrophobic wall protein set.

Experiment 3: <u>Distribution of proteins recognized by 6C5 and 5F8</u>

RCDs of hydrophilic yeast and germ tubes were compared with hydrophobic cells (Fig. 1). The three monoclonal antibodies recognized similar proteins from all three cell types. 5D8 recognized additional proteins in germ tubes (Fig. 1, lane 2) and hydrophilic cells (Fig. 1, lane 3). Indirect immunofluorescence assays were carried out to determine if the antigens recognized by the MAb were detectable on the surface of intact cells. The three antibodies appeared to bind solely to the hydrophobic germ tubes (Fig. 2). The patterns of binding, however, were different. 6C5 gave a punctate pattern of signal while the binding of 5F8 occurred over the entire germ tube surface. Binding of 5D8 was seen along the entire germ tube surface, but staining appeared to be more intense at the hyphal tips (Fig 2). IFA experiments carried out on ungerminated yeast cells showed that the signal was stronger on the surface of hydrophobic yeasts compared to hydrophilic cells. However, the signal on either type of yeast cell was much weaker than the signal observed on germ tubes (not shown). The sum of these results indicate that these three proteins are present in the walls of hydrophilic and hydrophobic yeast cells and germ tubes, but are more exposed or more abundant, on the surface of germ tubes.

The monoclonal antibodies were used to probe blots of wall proteins extracted from several species of *Candida*. As already seen by western blots and IFA, the wall of C. *albicans* contains all three proteins. *Candida tropicalis* cell wall extracts contained proteins which reacted with 6C5 and 5D8, however the 6C5-reactive protein was 54 kDa rather than 38 kDa. *C. kefyr* cell wall preparations contained proteins which bound 5F8 and 6C5. In these extracts, 5F8 also recognized a 59 kDa and a 55 kDa protein. The protein recognized by 6C5 was 36 kDa rather than 38 kDa. None of the three antibodies recognized proteins from lyticase digests of cell walls from *C. glabrata*, *C. guilliermondii*, *C. parapsilosis*, or *C. krusei*. One possibility is that the epitope, rather than the protein per se, is not present in the other *Candida* species. This may be the case for some of the proteins, but not all. Some preliminary results have indicated that the 40 kDa protein recognized by 5F8 is present in all the *Candida* species examined, but that additional extraction methods are required for its release. This differential expression,

even though it may be limited to epitopes, among the various *Candida* species reinforced our interest in these three proteins and our efforts towards their characterization.

Experiment 4: **ECM protein binding to yeast proteins**

Evidence that Ln and Fn bind to multiple C. *albicans* cell wall proteins has been previously shown (Bouchara *et al.*, 1990; Glee *et al.*, 1996; Lopez-Ribot *et al.*, 1994). Similar binding studies are included here for comparison (Fig. 3, lanes 1 & 3). Blotted RCD proteins were probed with either Ln or Fn followed by α-Ln or α-Fn, respectively. Both Ln and Fn bound to multiple cell wall proteins (Fig. 3, Ln and Fn, respectively). Of particular interest were proteins that migrated similarly to the proteins recognized by 5D8, 6C5 and 5F8 (Fig. 3, arrowheads).

Experiment 5: **Interaction between the wall proteins and ECM proteins**

To determine if the proteins recognized by the MAb bound ECM proteins themselves or if they were simply co-migrating with distinct ECM binding proteins, the ability of the MAb to block binding of intact cells to Fn and Ln was investigated. Pretreatment of a mixture of yeasts and germ tube initials with the antibodies significantly decreased the binding of cells to immobilized ECM proteins relative to untreated cells, shown as a decrease in relative binding rather than zero (Fig. 4). Pretreatment of cells with irrelevant antibody or control medium also resulted in a decrease of cell attachment, possibly due to steric effects from protein nonspecifically adsorbed to the cell surface. Although 5F8 significantly reduced cell adhesion to Ln relative to control (Fig. 4d), it did not reduce cell adhesion to Fn (Fig. 4c). The decrease in cell binding to both Fn and Ln due to pretreatment of cells with either 6C5 or 5D8 was greater than that due to control (Fig. 4a & b). From these results, we conclude that the proteins recognized by the MAb are ECM-binding proteins. Furthermore, the cell adhesion results relative to the irrelevant antibody controls also indicate that the interaction of the 38 and 40 kDa proteins with Fn and Ln has some degree of specificity

Experiment 6: **Binding Assay Under Shear Conditions**

Cell surface hydrophobicity (CSH) status influences the attachment of *Candida albicans* to various host tissue sites. CSH has also been implicated as a factor in adhesion of yeast cells to endothelial cells when tested in static adhesion assays. In order to simulate physiological shear forces present during hematogenous dissemination, we are using an in vitro shear assay to investigate C. *albicans* adhesion to human umbilical vein endothelial cells (HUVEC). Initial studies demonstrated that hydrophobic yeast bound more under shear that hydrophilic cells to interleukin-1β activated HUVECs. Using the in vitro shear assay, we tested the adhesion blocking ability of various monoclonal antibody reagents against *Candida* hydrophobic proteins. HUVEC monolayers were grown on the lumenal surface of capillary tubes, activated with interleukin- 1β and flow established with Hepes-buffered, Hank's balanced salts solution with Ca^{2+} and Mg^{2+} containing 5% human serum to simulate shear forces in a capillary vessel. *C. albicans* isolates were cultured in defined medium at 23 °C to establish hydrophobic yeast cell populations. Washed yeast cells were suspended in loop medium alone, MAb or control antibodies and assayed for adherence under shear (8-15 minute period, 1-2 dynes/ cm^2). Adherent yeast were counted from 10-15 random fields per monolayer and the average number of heterotypic binding events (*Candida*-HUVEC) and homotypic binding events (*Candida-Candida*) was determined. MAb 6C5, which recognizes a 38 kDa hydrophobic protein in that is more abundant on C. *albicans* hyphal surfaces than on hydrophobic yeasts, significantly reduced both kinds of binding events when compared to control conditions. The results indicate that blocking hydrophobic proteins on the surface of C. *albicans* yeast cells can decrease adhesion events occurring under shear.

A). C. *albicans* isolates and culture conditions: C. *albicans* was cultured aerobically in 0.055 M sodium phosphate (pH 7.2) buffered yeast nitrogen base plus amino acids (Difco) containing 2% glucose (YNB2G). Yeast cells were harvested, washed three times in cold sterile d-H_2O, counted, and the cell surface hydrophobicity (CSH) assessed by the hydrophobic microsphere assay (13). Yeast cells grown to stationary phase at 23 °C were hydrophobic (CSH ≥95%). Yeast aliquots were held on

ice as pellets and used within 4 hours. The yeast populations were also assessed for their sphere to cell unit ratios (S:CU), which is a measurement reflecting the abundance of singlet blastoconidia. For example, a mother daughter combination would be 2 spheres, but 1 contiguous cell unit. The S:CU values of ≤ 2:1 reflect stationary phase yeast cultures and were important for establishing the amount of *Candida-Candida* adhesion observed in these assays.

B). Anti-hydrophobic protein antibodies and control antibodies: Table 1 shows the anti-hydrophobic protein antibodies and control antibodies used. Mab 6C5 and Mab EL246 preparations were produced (Ligocyte Pharmaceuticals, Inc. Bozeman, Mont.) in serum-free medium (HB 101 Liquid Kit, Irvine Scientific, Santa Ana, CA) and concentrated by ammonium sulfate precipitation followed by exhaustive dialysis against Dulbecco's phosphate-buffered saline (DBPS, Sigma Chemical Co.) Mab 5D8, 5F8, 1C1 were ascites preparations produced at the University of Virginia Hybridoma Facility. Mab S10, which recognizes a group B streptococcal carbohydrate epitope, was an ascites preparation given as a gift from Dr. Seth Pincus (Montana State University, Bozeman, MT).

Table 1. Antibody reagents.

Antibody	Isotype, preparation	Antigen specificity	Source
MAb 6C5	IgG2a, serum free	38 kDa hydrophobic protein, C *albicans*	(14)
MAb 5F8	IgM, ascites	37 kDa hydrophobic protein, C *albicans*	(13a)
MAb 5D8	IgG2a, ascites	40 kDa hydrophobic protein, C *albicans*	(13a)
MAb lCl	IgM, ascites	~41 kDa hydrophobic protein, C. *albicans*	(13b)
EL246	IgG I, serum free	E- and L-selection	(15)
MAb S1O	IgM, ascites	Group B streptococcal carbohydrate epitope	(16)

C). Mammalian cells and growth conditions: Human umbilical vein endothelial cells (HUVECS) were harvested and prepared as previously described (15) or obtained commercially (Clonetics, San Diego, CA). Briefly, passage 4 or less HUVECs were grown 48 h to confluency on the lumenal surface of sterile glass capillary tubes (1.36 mm dia. x 2 cm) in endothelial-cell growth medium (EGM, Clonetics, San Diego, CA). For most assays, HUVEC monolayers were activated by incubation with 10 ng/ml interleukin- 1β (IL- 1β) for 1 h, rinsed, and EGM replaced for 2 h before the monolayer was utilized. The activations were performed on a staggered schedule to insure consistent intervals between treatment and use of the monolayers. Only capillary tubes having satisfactory monolayer development for approximately 70% of the tube length were utilized.

D). Shear Apparatus: The recirculating loop was prepared by connecting silicone tubing (1.5 mm i.d., 105 cm long with ~3 ml capacity) to each end of the capillary tube to form a closed system. A three-way stopcock and two extension sets were spliced into the loop and the tubing connected to a peristaltic pump that was calibrated to establish particular flow and shear force conditions. A Nikon inverted scope was equipped with phase contrast optics, a heated mechanical stage, and high resolution video apparatus. Adhesion assays were recorded continuously to provide off-line data analysis.

E). In vitro shear assay: Assays were performed in HEPES-buffered, Hank's balanced salts solution (plus Ca^{2+}/Mg^{2+}) containing 5% human serum. Final *Candida* concentrations of either 1×10^7 spheres/ml or 5×10^6 spheres/ml were utilized for adhesion assays involving yeast and HUVECs. Aliquots of *Candida* cells were suspended in 1 ml loop medium with or without anti-*Candida* or control antibodies at 60 μg/ml for 10 minutes on ice prior to injection into the loop under high flow rates (4-5 dynes/cm^2). Video recording was initiated and after 1 minute, the flow rate was adjusted down to 1-2 dynes/cm^2. At 8 - 12 minutes post-injection, the HUVEC monolayer was scanned by stopping at non-overlapping fields of view along the length of the capillary

tube. For each field of view, the microscope was adjusted through multiple focal planes to insure distinction of yeast bound to the HUVEC surface.

F). Data analysis: Video records were utilized to assess the number of adhesion events in each assay. At least 10 fields of view were analyzed for each assay. Different fields of view were chosen in the 8-12 minute window by using the internal time stamp per frame. SigmaStat v2.0 was utilized for statistical analysis of the experiments that were performed in duplicate.

Two types of binding events were scored in the *Candida* adhesion studies: 1). Heterotypic binding (*Candida* - HUVEC events) were counted as the number of foci of attached yeast per field. The average number of *Candida* foci per n fields was calculated for each assay; and 2). Homotypic binding (*Candida* - *Candida*) binding, expressed as ranked data to indicate how many blastoconidia or spheres were attached at each focus in the field of view. Rank sizes were chosen as 1, 2, 3, 4, 5-9, 10-15, and >16 blastoconidia attached per focus. Foci of 3 or more attached blastoconidia were considered the result of homotypic binding events based on the sphere to cell unit ratios of the *Candida* populations used for these shear experiments.

The relative contribution by ranks of 1, 2, and 3 or more to the average number of foci per field for each assay was determined to assess changes in homotypic binding events. For some assays, the average total number of blastoconidia per field for the assay was estimated from ranked data counts.

i). MAb 6C5 inhibits hydrophobic yeast cell binding to activated HUVECs:

Results of the in vitro shear analysis show that MAb 6C5 pretreatment of C. *albicans* caused significant inhibition of binding to IL- 1β activated endothelial cells compared to control yeast only (P < .001, t-test) and control MAb EL246 treatment (P < .00 1, t-test). The graph in Figure 5 shows the heterotypic binding events (average number of foci/field) for the assay. Table 2 shows the average number of total blastoconidia binding to the activated endothelium for different treatments. The table also shows percentage values for contributions by groups of 3 or more blastoconidia to the average

number of yeast foci per field. Homotypic binding was also influenced as shown in Table 2 for percentages reflecting the number of foci with 3 or more blastoconidia attached. Treatment with Mab 6C5 shows inhibition of homotypic adhesion events of *C. albicans* in that the total numbers of blastoconidia and percent contribution values are decreased.

Table 2. Homotypic binding events

Sample (number of fields)	**Average number of total blastoconidia per field**	**Percent of foci per field having ≥3 blastoconidia**
Yeast only (n = 12)	111.6	41.2
Yeast + MAb 6C5 (n = 15)	33.6	32.9
Yeast + MAb EL246 (n + 13)	73.4	39.5

Student t-test results for control yeast versus pre-treatment with EL246 in this assay was P = .04. We are cautious in interpreting the value as significant in light of other data (Table 3) in which control versus EL246 treatment was not significantly different.

<u>**ii). Influence of other anti-hydrophobic antibodies on the binding of *C. albicans* to activated HUVECs under physiological shear:**</u> Other anti-hydrophobic antibodies influence C. *albicans* binding to IL-1β activated HUVECs. A mixture of MAb 5F8, 5D8, and 1C1 ascites was compared with other antibody treatments for inhibition of hydrophobic yeast cell binding to activated HUVECs. MAb S10, which recognizes group B streptococcal carbohydrate antigens, was included as an irrelevant ascites control. EL-246 is a control antibody which blocks neutrophil selectin-mediated binding events with activated HUVECs. Table 3 shows that MAb 6C5 shows significant inhibition compared to yeast only control, and that the mixture of three other anti-hydrophobic protein antibodies (5F8, 5D8 and 1C1) produced significant inhibition compared to control ascites treatment.

TABLE 3. **Statistical analysis** - Student t-test utilized except where noted

Sample	Yeast only control	Control MAB S10 ascites
MAb 6C5	P <.001	
MAb EL246	P not significant (*)	
MAbs 5F8+5D8 + 1C1 ascites mix	P = .002	P = .022

*(Kruskal-Wallis ANOVA on ranks, EL246 data not normally distributed)

iii). Germination was inhibited in the presence of activated HUVECs under physiological shear: In two experiments, attached hydrophobic yeast cells were monitored for morphogenic changes over a two hour period. In both instances, less than 5% of the *Candida* cells in the recirculating loop (either in the bulk flow or attached to the endothelial surface) made germ tubes. The yeast cells incubated in loop medium alone at 37°C showed germ tube initials by 45 min (approx. 80%). It is not known whether the endothelial cells are producing a germination inhibitor or whether physiologic flow suppresses germination.

iv). Summary of results of Example 6: The mAb 6C5 recognizes a 38 kDa hydrophobic cell wall protein of the C *albicans* and inhibited attachment of hydrophobic yeast to activated human endothelial cells under physiological shear. The antibodies against other small hydrophobic cell wall proteins show inhibition activity in the *in vitro* shear experiments. A mixture of MAb 5F8 5D8, and 1C1 ascites significantly inhibited hydrophobic C. *albicans* binding to activated endothelium. An unexpected result was inhibition of germination in the presence of activated HUVECs under conditions of physiologic shear.

Example 7: Treatment of *Candida* Infection in Human Patients

For the treatment of disseminated disease, patients who develop evidence of disseminated disease should receive the antibody i.v. or i.p. or i.m., alone or in combination with other antifungal agents. For the prevention of candidiasis, high-risk patients should be identified, *e.g.*, those who will undergo abdominal surgery, open heart surgery, kidney transplants, bone marrow transplants, receive indwelling catheters, corticosteriods, broad spectrum antibiotics), the antibody pharmaceutical compositions described above should be administered i.v. or i.m. prior to the procedure. For the treatment of vaginal *Candida* infections, the antibody pharmaceutical compositions described above are administered intravaginally, as well as i.v. or i.p. or i.m., alone or in combination with other antifungal agents.

Example 8: Use of Test Kits to Detect *Candida* Infection

The antibodies as described above are used in a capture antigen format to capture *Candida* antigen in the serum or vaginal secretions from an infected patient. Such kits are further prepared with agents to detect the binding of the antibody to such antigens.

It should be understood that the foregoing discussion and examples present merely present a detailed description of certain preferred embodiments. It therefore should be apparent to those of ordinary skill in the art that various modifications and equivalents can be made without departing from the spirit and scope of the invention. All articles, patents and patent applications that are identified above are incorporated by reference in their entirety. Also incorporated by reference is a co-pending application filed on even date herewith, entitled "Vascular and Mucosal Shear Analysis System for Host-Pathogen Interactions" with Attorney Docket No. 48984-5001.

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Smith, P. K., Krohn, R. I., Hermanson, G. T., Mallia, A. K., Gartner, F. H., Provenzano, M. D., Fujimoto, E. K., Goeke, N. M., Olson, B. J. & Klenk, D. C. (1985). Measurement of protein using bicinchoninic acid. Anal. Biochem. 150, 76-85.

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Tronchin, G., Bouchara, J. P. & Robert, R. (1989). Dynamic,changes of the cell wall surface of *Candida*, *albicans* associated with germination and adherence. Eur. J. Cell. Biol. 50, 285-290.

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We claim:

1. A monoclonal antibody that specifically binds to an epitope of a hydrophobic cell wall protein of a yeast from the *Candida* genus and inhibits the binding of the protein to a tissue of a mammalian host.

2. The antibody of claim 1, wherein the hydrophobic cell wall protein mediates adhesion of the yeast to the tissue.

3. The antibody of any of claims 1 and 2, wherein the hydrophobic cell wall protein mediates adhesion of the yeast to the tissue under conditions of physiological shear present in the tissue.

4. The monoclonal antibody of claim 3, which is selected from the group consisting of 5F8, 5D8, 1C1 and 6C5 antibodies and fragments or mixtures thereof.

5. The monoclonal antibody of claims 3, wherein the antibody is selected from the group consisting of IgG, IgA and IgM.

6. The monoclonal antibody of claim 3, wherein the yeast is selected from the group consisting of *C. albicans, C. kefyr, C. lipolytica, C. rugosa, C. stellatoidea* and *C. tropicalis* and strains thereof.

7. The monoclonal antibody of claim 3, wherein the antibody is selected from the group consisting of 5F8, 5D8, 1C1 and 6C5 antibodies and fragments and mixtures thereof.

8. The antibody of claims 3, wherein the molecular weight of the hydrophobic cell wall protein, as determined by SDS-PAGE, is less than about 90 kDa.

9. The antibody of claim 8, wherein the molecular weight of the hydrophobic cell wall protein, as determined by SDS-PAGE, is between about 20-70 kDa.

10. The antibody of claim 9, wherein the molecular weight of the hydrophobic cell wall protein, as determined by SDS-PAGE, is about 37 kDa, about 38 kDa, about 40 kDa or about 41 kDa.

11. The antibody of claim 9, wherein the yeast is *Candida tropicalis*.

12. The antibody of claim 11, wherein the molecular weight of the hydrophobic cell wall protein, as determined by SDS-PAGE, is about 40 kDa or about 54 kDa.

13. The antibody of claim 9, wherein the yeast is *Candida kefyr*.

14. The antibody of claim 13, wherein the molecular weight of the hydrophobic cell wall protein, as determined by SDS-PAGE, is about 36 kDa, about 55 kDa or about 59 kDa.

15. The antibody of claim 3, wherein the antibody is a human antibody, a chimeric antibody, or a humanized antibody.

16. An antigen binding fragment of the monoclonal antibody of claim 3, wherein said fragment is selected from the group consisting of a Fv fragment, a Fab fragment, a Fab' fragment, and a $F(ab')_2$.

17. The antibody of claim 3, wherein said antibody is protective against disseminated *Candida* infection in the host.

18. The antibody of claim 3, wherein said antibody is protective against mucocutaneous *Candida* infection in the host.

19. A pharmaceutical composition comprising the antibody claim 3 together with pharmaceutically acceptable carrier and excipients.

20. The pharmaceutical composition of claim 19, formulated for systemic administration.

21. The pharmaceutical composition of claim 19, formulated for topical administration.

22. The pharmaceutical composition of claim 19, formulated as an aerosol.

23. The pharmaceutical composition of claim 19, further comprising one or more other therapeutic agents.

24. The pharmaceutical composition of claim 23, wherein said one or more other therapeutic agents is an antifungal agent.

25. The pharmaceutical composition of claim 23, wherein the antifungal agent is selected from the group consisting of amphotericin B, fluconazole, new generation azoles and mixtures thereof.

26. A method of treating candidiasis in a subject comprising the step of administering to a subject a therapeutically effective amount of the pharmaceutical composition of claim 19.

27. The method of claim 26, wherein the therapeutically effective amount is an amount which is effective to inhibit the binding of a hydrophobic cell wall protein of a yeast from the *Candida* genus to a tissue of a mammalian host.

28. The method of claim 26, wherein the pharmaceutical composition is effective to treat or prevent disseminated candidiasis.

28. The method of claim 26, wherein the pharmaceutical composition is effective to treat or prevent mucocutaneous candidiasis.

29. The method of any of claim 25, wherein the yeast is selected from the group consisting of *C. albicans, C. kefyr, C. lipolytica, C. rugosa, C. stellatoidea* and *C. tropicalis* and strains thereof.

30. A diagnostic kit comprising the antibody of claim 3, together with a reagent for detecting binding of the antibody to a hydrophobic cell wall protein of a yeast from the *Candida* genus.

31. A hybridoma cell that expresses the antibody of any of claim 3.

32. The antibody of any of claims 1 and 2, wherein the antibody binds to the yeast under conditions of physiological shear present in a host tissue.

33. The antibody of claim 32, wherein binding of the antibody blocks attachment of the yeast to the host's tissue or cells.

34. A hydrophobic cell wall protein of a yeast of the *Candida* genus which mediates adhesion of the yeast to the tissue of a mammalian host, wherein the molecular weight, as determined by SDS-PAGE, is about 36 kDa, 38 kDa, 40 kDa, 41 kDa, 54 kDa, 55 kDa or 59 kDa.

35. The protein of claim 34, wherein the host tissue is selected from the group consisting of endothelial cells, epithelial cells or extracellular matrix proteins.

36. The protein of claim 35, wherein the protein is capable of binding to the host tissue under physiological shear conditions present in the tissue.

Figure 1

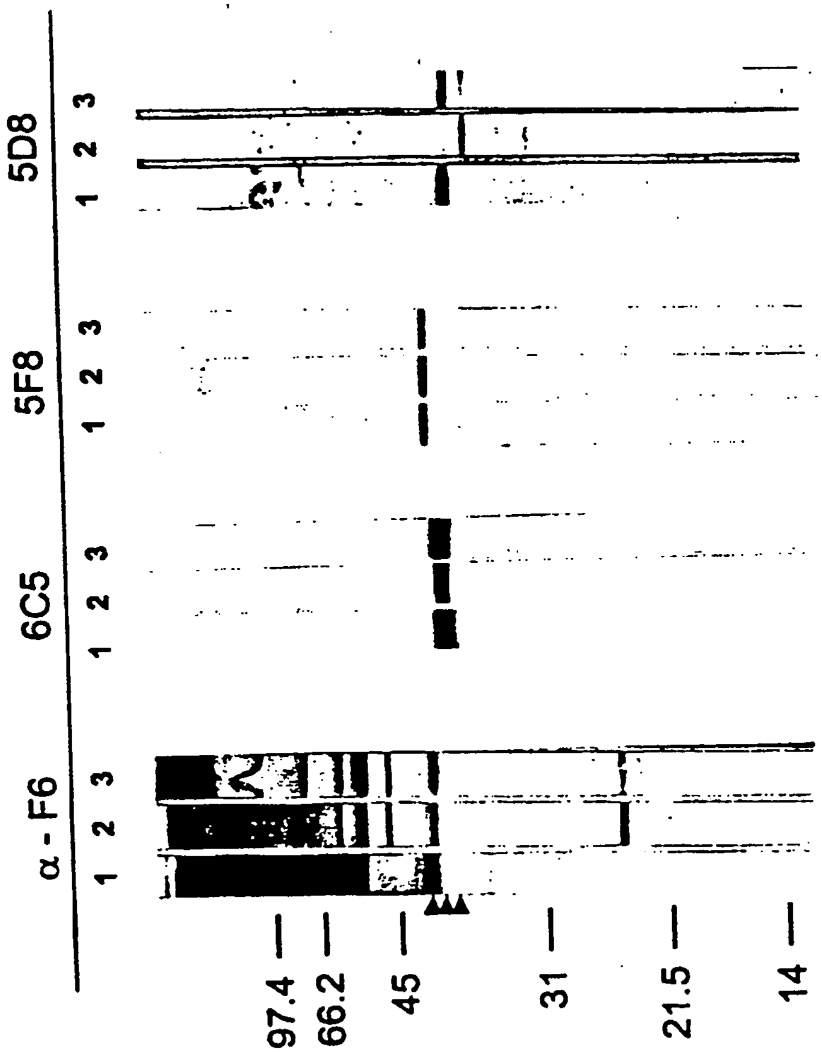


Figure 2

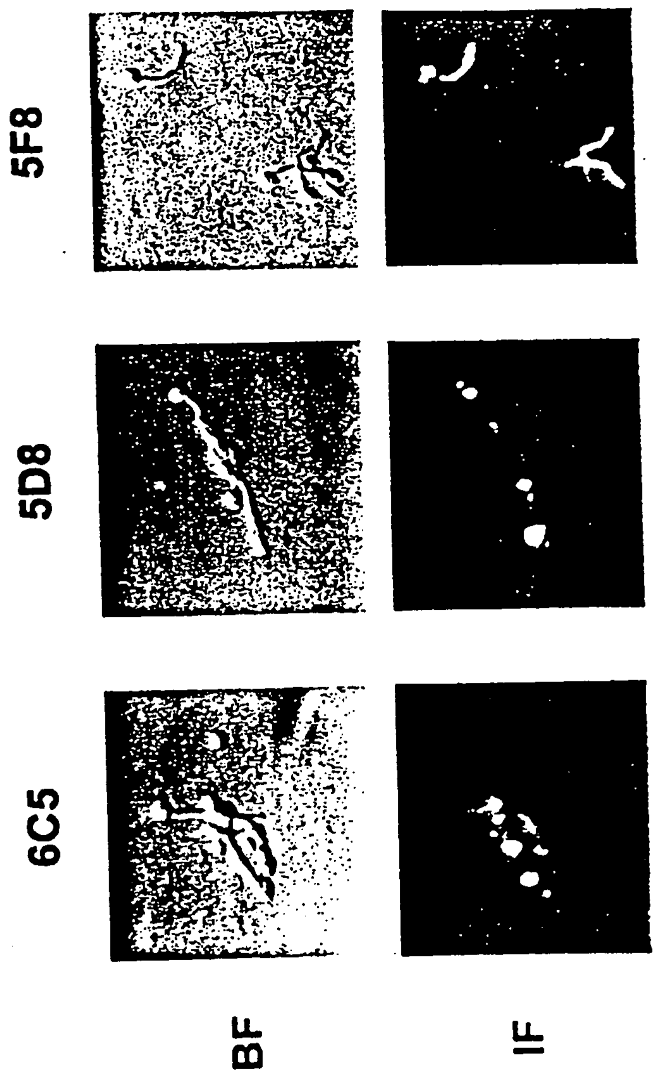
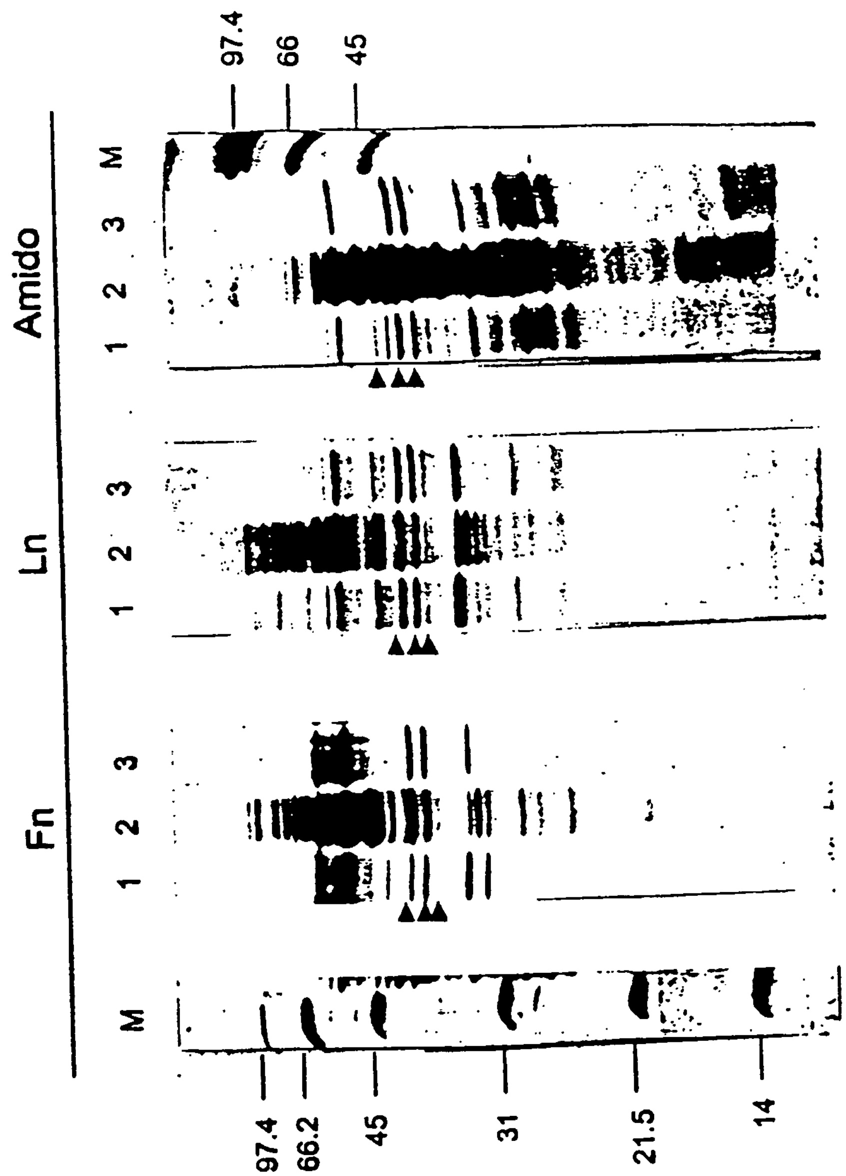
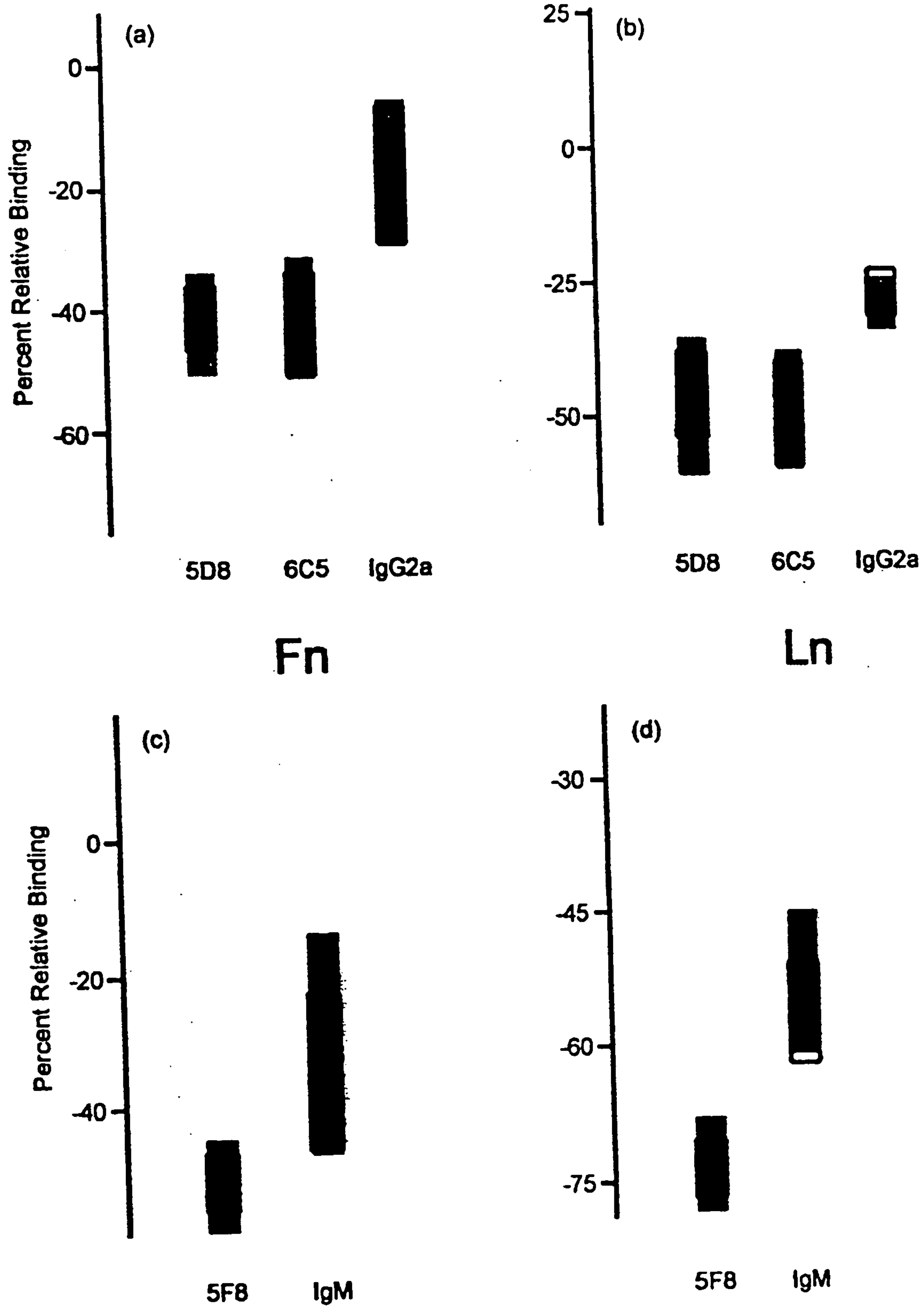


Figure 3




(a)
Percent Relative Binding
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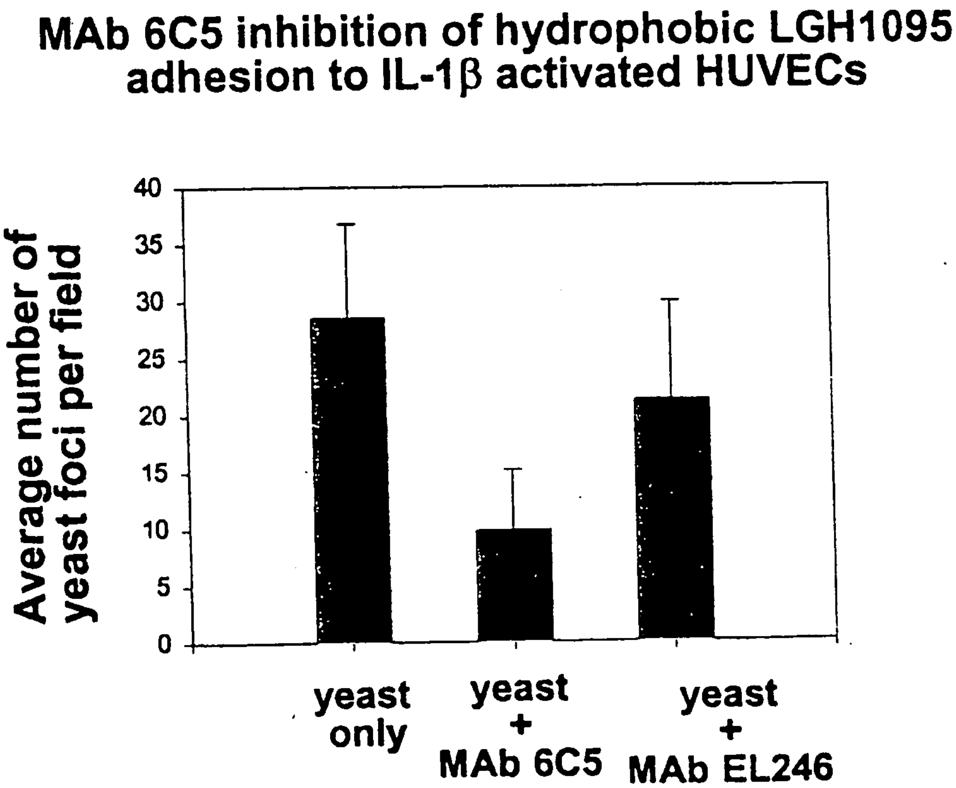
-20
-40
-60
5D8
6C5
IgG2a
Fn
(b)
25
0
-25
-50
5D8
6C5
IgG2a
Ln
(c)
Percent Relative Binding
0
-20
-40
5F8
IgM
(d)
-30
-45
-60
-75
5F8
IgM

Figure 4

Figure 5


MAb 6C5 inhibition of hydrophobic LGH1095
adhesion to IL-1β activated HUVECs
Average number of yeast foci per field
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35
30
25
20
15
10
5
0
yeast only
yeast + MAb 6C5
yeast + MAb EL246

Figure 6

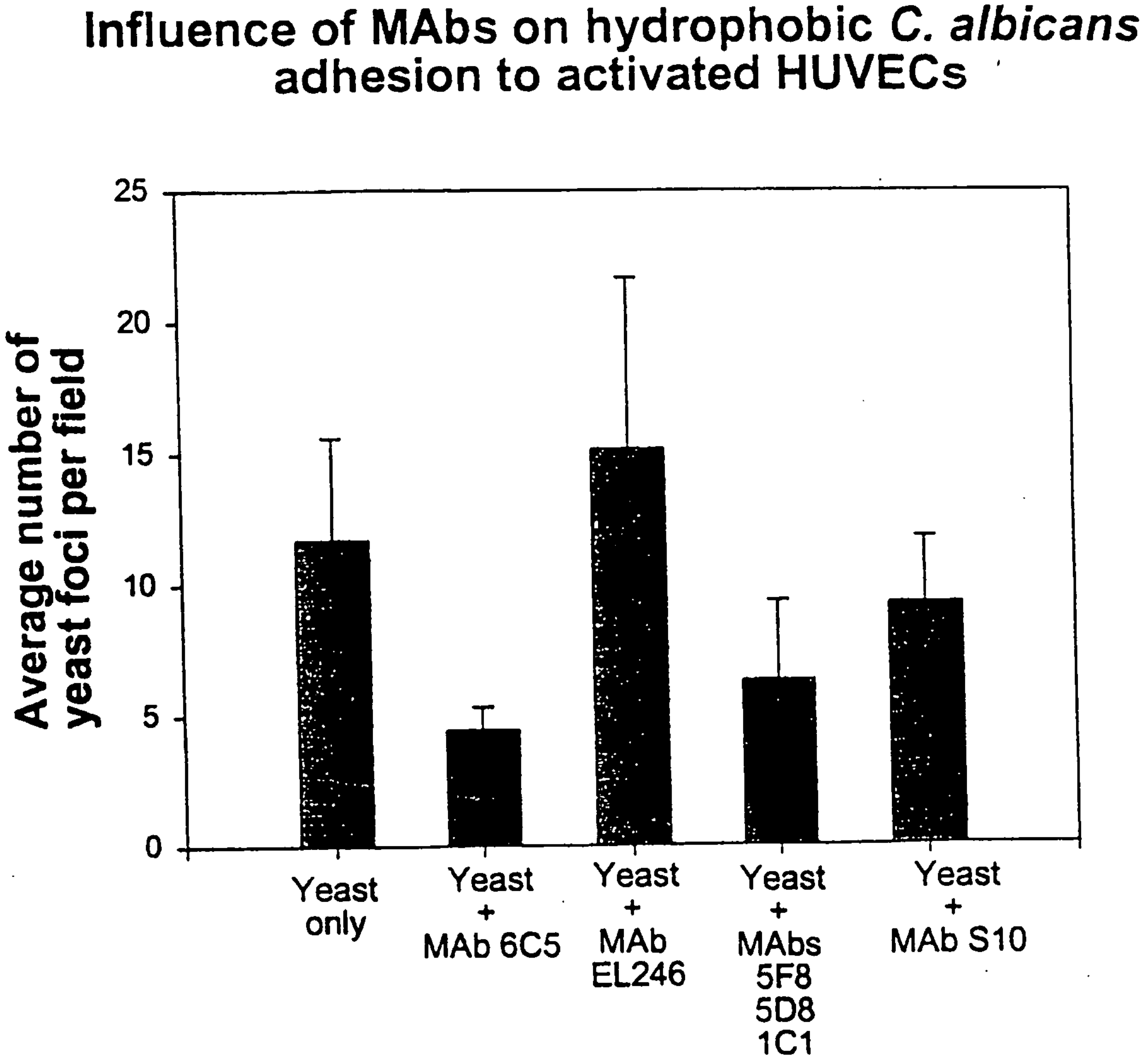
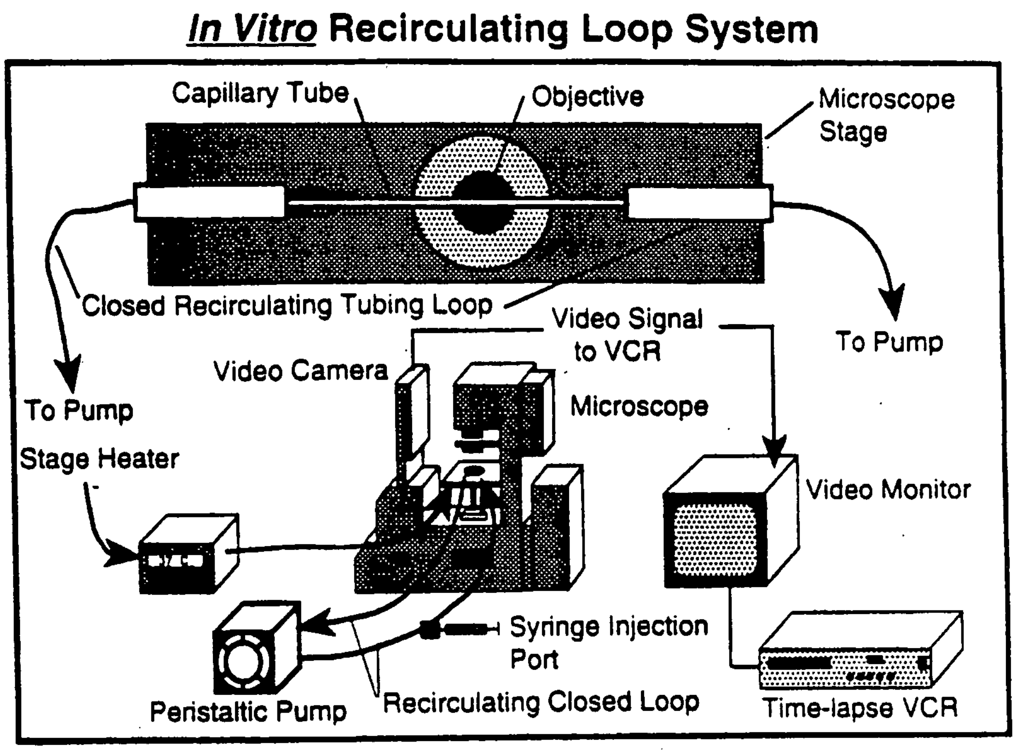

Influence of MAbs on hydrophobic C. albicans adhesion to activated HUVECs
Average number of yeast foci per field
25
20
15
10
5
0
Yeast only
Yeast + MAb 6C5
Yeast + MAb EL246
Yeast + MAbs 5F8 5D8 1C1
Yeast + MAb S10

Figure 7


In Vitro Recirculating Loop System
Capillary Tube
Objective
Microscope Stage
Closed Recirculating Tubing Loop
Video Signal to VCR
To Pump
Video Camera
Microscope
To Pump
Stage Heater
Video Monitor
Syringe Injection Port
Peristaltic Pump
Recirculating Closed Loop
Time-lapse VCR

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US00/04447

A. CLASSIFICATION OF SUBJECT MATTER

IPC(7) :A61K 39/395; C12N 5/16
US CL :424/ 130.1, 134.1, 141.1, 152.1; 435/326
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 424/ 130.1, 134.1, 141.1, 152.1; 435/326

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

WEST, MEDLINE, BIOSIS, CAPLUS

search terms: cell wall protein, antibodies, candida, treatment,

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	US 5,641,760 A (YU ET AL.) 24 June 1997 (24-06-97), see entire document.	1-2, 34-36
Y	US 5,578,309 (CUTLER ET AL.) 26 November 1996 (26-11-96), see entire document.	1-2, 34-36
Y	MASUOKA et al. Inhibition of Candida albicans attachment to extracellular matrix by antibodies which recognize hydrophobic cell wall proteins. FEMS Immunology and Medical Microbiology. 1999. Vol 24, No. 4, pages 421-429. See entire document.	1-2, 34-36

[X] Further documents are listed in the continuation of Box C. [ ] See patent family annex.

* Special categories of cited documents:

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
12 MAY 2000	27 JUL 2000

Name and mailing address of the ISA/US	Authorized officer
Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231	Terry J. Dey for ROBERT A. ZEMAN
Facsimile No. (703) 305-3230	Telephone No. (703) 308-0196

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	ANGIOLELLA et al. Identification of a glucan-associated enolase as a main cell wall protein of Candida albicans and an indirect target of lipopeptide antimycotics. Journal of Infectious Diseases. March 1996, Vol. 173, No. 3, pages 684-690. See entire document.	1-2, 34-36
Y	HAN et al. Biochemical characterization of Candida albicans epitopes that can elicit protective and nonprotective antibodies. Infection and Immunity. October 1997, Vol. 65, No. 10, pages 4100-4107. See entire document.	1-2, 34-36
Y	LEE et al. The Effects of monoclonal antibodies against iC3b receptors in mice with experimentally induced disseminated candidiasis. Immunology, September 1997, Vol. 92, No. 1, pages 104-110. See entire document.	1-2, 34-36
Y	MASUOKA et al. Cell wall protein mannosylation determines Candida albicans cell surface hydrophobicity. Microbiology. September 1997, Vol. 143, Pt. 9, pages 3015-3021. See entire document.	1-2, 34-36

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US00/04447

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This international report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. ☐ Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. ☒ Claims Nos.: 3-33
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

Please See Extra Sheet.

1. ☒ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

☐ The additional search fees were accompanied by the applicant's protest.

☒ No protest accompanied the payment of additional search fees.

Form PCT/ISA/210 (continuation of first sheet(1)) (July 1998)★

BOX II. OBSERVATIONS WHERE UNITY OF INVENTION WAS LACKING
This ISA found multiple inventions as follows:

This application contains the following inventions or groups of inventions which are not so linked as to form a single inventive concept under PCT Rule 13.1. In order for all inventions to be searched, the appropriate additional search fees must be paid.

Group I, claim(s) 1-33, drawn to antibodies, pharmaceutical compositions comprising said antibodies, a hybridoma, a diagnostic kit and methods of treatment using said compositions.

Group II, claim(s) 34-36, drawn to cell wall proteins.

This application contains claims directed to more than one species of the generic invention. These species are deemed to lack Unity of Invention because they are not so linked as to form a single inventive concept under PCT Rule 13.1. In order for more than one species to be searched, the appropriate additional search fees must be paid. The species are as follows:

Group II cell wall proteins of: 36 kDa; 38 kDa; 40 kDa; 42 kDa; 41 kDa; 54 kDa; 55 kDa; and 59 kDa.

The inventions listed as Groups I and II do not relate to a single inventive concept under PCT Rule 13.1 because, under PCT Rule 13.2, they lack the same or corresponding special technical features for the following reasons:

The inventions listed as Groups I and II do not relate to a single general inventive concept under PCT Rule 13.1 because, under PCT Rule 13.2, they lack the same or corresponding special technical features for the following reasons:

Pursuant to 37 C.F.R. 1.475(d), the ISA/US considers that where multiple products and processes are claimed, the main invention shall consist of the first invention of the category first mentioned in the claims and the first recited invention of each of the other categories related thereto. Accordingly, the main invention (Group I) comprises the first-recited product, antibodies. Further pursuant to 37 C.F.R. 1.475(d), the ISA/US considers that any feature which the subsequently recited products and methods share with the main invention does not constitute a special technical feature within the meaning of PCT rule 13.2 and that each of such products and methods accordingly defines a separate invention.

The species listed above do not relate to a single inventive concept under PCT Rule 13.1 because, under PCT Rule 13.2, the species lack the same or corresponding special technical features for the following reasons: Each of the listed species is required to distinctly different chemical, physiological, biological and immunological properties and thus cannot be said to share the same or corresponding special technical feature.